Avian Diseases

FEBRUARY 1961

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JOHN PAUL DELAPLANE,
DECEMBER 4, 1906—SEPTEMBER 22, 1957

At the third annual meeting of the American Association of Avian Pathologists, held in Denver, Colorado, August 15, 1960, the outgoing president, Dr. Henry Van Roekel, University of Massachusetts, Amherst, Massachusetts, closed the meeting by presenting the association with a gavel, which was accepted by the incoming president, Dr. Stephen B. Hitchner, Berlin, Maryland. The American Association of Avian Pathologists is deeply grateful to Dr. Van Roekel, whose moving citation in memory of Doctor John P. Delaplane is reproduced herewith:

CITATION IN MEMORY

of

DOCTOR JOHN P. DELAPLANE

The American Association of Avian Pathologists is the realization of an idea and purpose first advanced by a group of workers engaged in poultry diseases at one of our outstanding Land-Grant colleges.

Among this group of workers, one individual in particular was responsible for initiating and formulating the purposes of our organization and how best it might serve those engaged in poultry disease work.

This individual had devoted his entire professional career towards serving the poultry industry. His keen insight, untiring efforts and unique ability to resolve difficult disease problems have been phenomenal. His scientific contributions in the field of poultry diseases have been outstanding and numerous. He was respected by his professional colleagues for his scientific curiosity, integrity, modesty, and professional ethics.

At this third annual meeting of the American Association of Avian Pathologists, I, as chairman of this meeting, with sincere humility and greatest of respect, wish to present to this organization this gavel which is inscribed "In Memory of John P. Delaplane, 1960."

H. VAN ROEKEL

August 15, 1960.

SUMMARY OF THE THIRD ANNUAL MEETING OF THE AMERICAN ASSOCIATION OF AVIAN PATHOLOGISTS, DENVER, COLORADO, AUGUST 15, 1960

THE third annual meeting was attended by 51 members. During the year the American Association of Avian Pathologists became incorporated as a nonprofit organization in the state of Delaware and has assumed legal and financial responsibility for the journal AVIAN DISEASES, official publication of the association.

The officers for 1960-61 are: Dr. Stephen B. Hitchner, President; Dr. Henry E. Adler, Vice-President; Dr. Morris S. Cover, Secretary-Treasurer; Western District Member of the Board of Directors, Dr. L. G. Raggi (term expires 1964). The other directors (and expiration of terms) are: Eastern District, P. P. Levine (1961); Southern District, A. I. Flowers (1962); Central District, M. S. Hofstad (1963).

The standing committees of the association for 1961 are: *Membership*: R. F. Gentry, Pennsylvania State University, University Park, Pennsylvania, chairman; C. A. Bottorff; W. J. Benton. *Nominating*: Glenn H. Snoeyenbos, Chairman; P. H. Seitz; C. Gale. *Resolutions*: Dennis Sykes, chairman; B. R. Burmester; C. H. Bigland.

Six special committees were appointed:

Veterinary Poultry Pathology Programs. This is a committee to survey educational opportunities and procedures in three general areas—(1) veterinary schools, (2) graduate training, (3) refresher or short course training not leading to a degree. Members continue to be: H. E. Adler, H. L. Chute, C. L. Nelson.

The report indicated that there is little uniformity in the teaching of avian diseases—less than half the schools give a laboratory section along with lectures. This deficiency is corrected to some extent by the poultry pathology assignment

in senior clinic. The duration of poultry clinics instruction varies from school to school.

Committee on Pullorum Diseases Eradication. The report of this committee stimulated a good deal of discussion. The proposals are to be submitted to the proper authorities in each of the states. Members continue to be: B. S. Pomeroy, chairman; J. E. Williams; D. E. Stover; L. C. Grumbles; H. Van Roekel.

Committee on Industrial Relations. The president, Dr. Hitchner, suggested that conducting periodic refresher courses for practitioners, diagnosticians, and those serving the poultry industry might both benefit the association and stimulate better understanding between the poultry industry and the veterinarians serving them.

The committee: E. L. Jungherr, chairman; C. W. Darby; J. Bivins; R. A. Bagley; K. Schaaf; J. R. E. Taylor, reported limited surveys among poultrymen in two widely separated areas on the adequacy of diagnostic service which indicated needs for improvement, especially in accuracy of diagnosis, timing of reports, and provision for emergency service. Representatives of other scientific disciplines feel there is inadeqate cooperation with the poultry pathologist and that the invisible barrier between the pathologists and the other disciplines should be broken down for the good of the industry. In many areas the physical set-up permits such cooperation but effective collaboration is yet to be developed.

Committee on Reporting Diseases and Terminology. This committee recommended making an annual report of various disease conditions diagnosed in different areas of the country showing prevalence, occurrence and distribution. A standard form for use by diagnostic laboratories for such reporting is being designed. Such a compilation would be of great value, especially to diagnosticians and researchers. Committee membership is C. I. Angstrom, chairman; Wilson Henderson; A. L. Kleckner; L. G. Raggi.

Mimeographed reports of the special committees have been circulated to the membership of the AAAP.

One other committee, *the Committee on Poultry Inspec-*

tion, stimulated considerable discussion with its report (not mimeographed). It reported on activities in three areas:

(a) Acting as liaison between the members of the AAAP and the inspection service, they reported on activities aimed at alleviating some of the growing pains of the inspection service, including changes in reporting methods, recently adopted, and data concerning training programs for the poultry meat inspection staff. The committee suggested urgent need for increased emphasis in the subject area in the veterinary schools, plus better support from the veterinary profession for adequate salaries of poultry meat inspectors.

(b) The committee urged cooperation between regional diagnostic laboratories and the poultry meat inspection service, pointing out that considerable organization must go into this relationship for the program to be meaningful.

(c) The committee recommended a study of the areas of research in avian pathology that may relate to the wholesomeness of poultry carcasses to make subsequent recommendations as to research needed. This committee continues to function. Members are Walter W. Sadler, chairman; C. I. Boyer; Daniel DeCamp; L. C. Grumbles; Dank Morris; and R. E. Omohundro.

AMERICAN ASSOCIATION

of

AVIAN PATHOLOGISTS

Fourth Annual Meeting

August 21, 1961

7:15 P. M.

Detroit, Michigan

THE CHEMOTHERAPEUTIC ACTIVITY OF 3,5-DINITROBENZAMIDE.*
I. AGAINST PARATYPHOID IN TURKEYS

M. MITROVIC,** P. H. MATISHECK, and L. C. LYNCH
Research Division, Dr. Salsbury's Laboratories, Charles City, Iowa

Received 10 Mar 1960

PARATYPHOID, infection caused by motile *Salmonella*, has long been recognized as one the most prevalent bacterial diseases of turkeys, and one of the most difficult to treat and control. This kind of infection is of great concern to the expanding turkey industry.

A survey of the incidence of paratyphoid in turkeys in northeastern Iowa and southern Minnesota, from January through December of 1955, revealed the disease in 59 flocks, totaling 181,960 birds. Mortality ranged from 1% to 66% (average 8.1%), the age at death varying from five days to adulthood. The highest incidence was in April. This survey emphasized the intensity of infection in a single area, which,

* Tristat is a trade name of the product containing 3,5-dinitrobenzamide developed by Dr. Salsbury's Laboratories, Charles City, Iowa.

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if projected to more extensive areas, indicates the infection-potential of this disease.

Eradication programs based solely on management and sanitation practices have failed to control the disease. The difficulties encountered have been related to the growing number of new serotypes and to the lack of effective medicaments.

The discovery of chemotherapeutic agents as sulfonamides, antibiotics, and nitrofurans has contributed to the control of paratyphoid. Such drugs, combined with sound management and sanitation practices, have often helped control the disease.

Morehouse and McGuire[1] reported on the use of 3,5-dinitrobenzamide (3,5-DNBA) and its N-substituted derivatives against coccidiosis in chickens. The present report deals with the chemotherapeutic activity of 3,5-DNBA against *Salmonella* in turkeys.

MATERIALS AND METHODS

In vitro sensitivity test. The plate-disc method was used throughout these tests. The discs were S and S No. 740-E discs (Carl Schleicher & Schuell Co., Keene, N.H.), diameter 12.5 mm, made of very pure highly absorbent paper for the assay of penicillin and other antibacterial substances. Acetone and methanol were used as solvents for the 3,5-DNBA. Amounts of 0.01, 0.1, 1.0, 5.0 and 10.0 mg of 3,5-DNBA per disc were applied, and the discs dried overnight.

One ml of a 24-hr tryptose broth culture of Salmonella sp., of various serotypes, containing about 5×10^8 bacteria per ml, was poured into a Pyrex baking dish (10×10 in.). To this was added 500 ml of properly cooled Bacto-tryptose agar, which was moderately agitated to mix the bacteria with the agar. After the agar was solidified, the filter discs containing 3,5-DNBA of appropriate concentrations were placed on the surface of the medium. The dish was incubated 24 hours at 37°C and inspected. The activity of the compound was assessed by the presence or absence of a zone of inhibition around the discs. Sterile disc controls were included. With this procedure, 47 Salmonella cultures representing 20 different serotypes were assayed *in vitro*.

Table 1. The effect of 3,5-DNBA in feed against *S. typhimurium*.

Test no.	% conc. of 3.5-DNBA	Total mort.[1] IM[2]	IUC[2]	UUC[2]	Age of poults
Medicated 3 days before infection					
1	0.10	5/15	13/17	0/11	1 day
2	0.10	2/10	4/10	0/10	1 day
3	0.10	1/12	5/10	0/10	1 day
4	0.075	2/10	5/10	0/10	1 day
Medicated at time of infection					
5	0.15	1/25	4/24	0/24	1 day
6	0.15	3/24	20/25	0/23	1 day
7	0.15	3/10	6/10	0/10	1 day
8	0.15	0/12	3/12	0/12	2 wks
9	0.10	1/24	4/24	0/24	1 day
10	0.075	1/12	3/12	0/12	2 wks
Medicated 1 day after infection					
11	0.15	8/15	12/15	0/15	1 day
12	0.15	9/24	20/25	0/25	1 day

[1] Numerator = number of poults dead; denominator = number of poults used.

[2] IM = infected-medicated; IUC = infected-unmedicated controls; UUC = uninfected-unmedicated controls.

In vivo efficacy tests. The experiments were conducted under strict isolation. Broad-Breasted Bronze turkey poults free of paratyphoid, one day to two weeks old, were used throughout the trials. The birds were kept in wire-floored electrically heated battery brooders and fed a turkey-growing mash. The 3.5-DNBA was incorporated in feed at 0.075%, 0.10% and 0.15%, properly mixed for even distribution. No other medicaments were included in the ration. Medication was given either three days before, simultaneously with, or one day after infection. In all these tests the birds were medicated for 14 days.

A 24-hr tryptose broth culture of *Salmonella typhimurium* with an approximate bacterial concentration of 5×10^8 cells per ml was used for exposure. Of this, 0.2 ml was introduced into the crop of each poult with a syringe through a blunt needle. The efficacy of 3,5-DNBA was based upon the difference in survival between the infected-medicated and infected-unmedicated controls. Following the trial, the birds were killed and observed for gross pathology (Table 1).

In vivo safety and palatability test. This experiment was conducted under strict isolation with one-day-old Broad-Breasted Bronze turkey poults. The poults were purchased from a hatchery free of paratyphoid. The birds were kept in wire-floored electrically heated battery brooders. A turkey-growing mash ration containing 3,5-DNBA 0.075% and 0.15% was fed to turkey poults for four weeks. The medicated or unmedicated feed and water were given *ad libitum.* Records were kept of body weight at weekly intervals, together with the feed consumption and feed conversion (Table 2). After the 4-week period the trial was terminated, the birds were necropsied and examined for gross pathology.

RESULTS AND DISCUSSION

In vitro sensitivity test. The results showed that 47 cultures of genus *Salmonella* representing 20 different serotypes in Groups A, B, C_1, C_2, D, E_1, E_2, and E. were sensitive to 3,5-DNBA at 1.0, 5.0, and 10.0 per disc. No sensitivity was detected at 0.01 and 0.1 mg per disc. The reason may be the poor solubility and diffusibility of 3,5-DNBA in the medium used. Even so, the assay showed 3,5-DNBA to be active *in vitro* against 20 different serotypes of *Salmonella.* The *Salmonella* serotypes tested (and number of isolates of each)

Table 2. The effect of 3,5-DNBA in feed on growth and palatability in the first four weeks of life.

Group	A	B	C
3,5-DNBA, % conc. in feed	0.075	0.15	.0
No. of poults	33	33	33
Mean weight (g): start	58	58	57.9
end	444.9	388.3	401.9
Mean weight gain (g)	386.9	330.3	344.0
Ratio of gain to that of C	1.11	0.96	1.0
Total feed consumed (kg)	23.08	26.85	26.95
Feed conversion	1.92	2.54	2.45
Ratio of feed conversion to that of C	0.78	1.04	1.0

were: *typhimurium* (8), *san diego* (2), *saint paul* (3), *reading* (3), *kaapstad* (2), *derby* (2), *california* (1), *bredeney* (1), *schwarzengrund* (2), *thompson* (2), *oranienburg* (2), *infantis* (2), *newport* (4), *muenchen* (2), *give* (2), *senftenberg* (1), *newington* (2), *anatis* (4), *heidelberg* (1), and *enteritidis* (1).

In vivo efficacy tests. The results are reported in Table 1. Tests 1, 2, and 3 showed that 3,5-DNBA, fed at the level of 0.10% to turkey poults three days before infection, effectively reduced mortality: 8/37 infected-medicated poults died (mort. 21%), in contrast with 22/37 infected-unmedicated (mort. 56%). Test 4 showed that 3,5-DNBA in feed at the level of 0.075% also reduced losses: 2/10 infected-medicated poults died (mort. 20%), compared with 5/10 infected-unmedicated (mort. 50%).

In tests 5, 6, 7, and 8, with 3,5-DNBA given at the therapeutic level of 0.15% at the time of infection, only 7/71 infected-medicated poults died (mort. 9%), compared with 33/71 infected-unmedicated (mort. 46%). Tests 9 and 10 showed that 3,5-DNBA administered in feed at the levels of 0.10% and 0.075% was effective in reducing mortality among the infected-medicated groups, 1/24 (mort. 4%) and 1/12 (mort. 8%), respectively, in comparison with the infected-unmedicated controls, 4/24 (mort. 16%) and 3/12 (mort. 25%).

In tests 11 and 12, with 3,5-DNBA administered in feed at the level of 0.15% 24 hours after infection, when the birds showed evident signs of disease, 17/39 infected-medicated poults died (mort. 44%), compared with 32/40 infected-unmedicated controls (80%).

The *S. typhimurium* strain was chosen for inoculation because it was the strain most often isolated from natural outbreaks in our laboratory. At the end of these trials *S. typhimurium* could be isolated from infected-medicated birds; the ratio of positive isolates, however, was much lower than from the infected-unmedicated poults (0-30%, vs. constantly 100%), this indicates that 3,5-DNBA at the levels used acted as a bacteriostat *in vivo*, possibly bactericidal in some instances.

The drug prevented death in one trial only, but considerably reduced the mortality from the virulent culture of *S. typhimurium* throughout the remaining trials. The compound, used at the above levels and for a period of 14 days, did not affect the turkey poults adversely.

Safety and palatability of 3,5-DNBA. The data in Table 2 show that 0.075% and 0.15% 3,5-DNBA fed poults for four weeks, from one day old, appeared palatable and safe. In fact, Group A gained 11% better than unmedicated controls, and Group B, fed at the higher level, gained 4% less. This difference, however, was not statistically significant. It should be pointed out that the medication lasted twice as long as that used in the infection trials.

ACKNOWLEDGMENTS

The authors thank Dr. P. R. Edwards, Communicable Disease Center, Chamblee, Georgia, for the serotyping of Salmonella.

SUMMARY

A new chemotherapeutic agent, 3,5-DNBA, inhibited 20 different Salmonella serotypes *in vitro*. *In vivo* efficacy data, under laboratory conditions, showed the antibacterial properties of 3,5-DNBA in feed at levels of 0.075%, 0.10% and 0.15% against a lethal inoculation of *S. typhimurium* in turkey poults. The compound reduced mortality when fed either three days before, simultaneously with, or one day after infection. A 28-day medication trial at levels of 0.075% and 0.15% showed only slight depression of turkey poults from 3,5-DNBA, indicating that the compound was safe and palatable.

REFERENCE

1. Morehouse, N. F., and W. C. McGuire. The use of 3,5-Dinitrobenzamide and its N-substituted Derivatives against Coccidiosis in chickens. Poultry Sci., *38*, 410-423, 1959.

FIELD TRIALS IN THE CONTROL OF GAPEWORM INFECTIONS IN PHEASANTS

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Received 13 May 1960

MOST of the interest in control of *Syngamus trachea* in domestic birds has centered on methods of destroying or removing the parasites from the trachea of their hosts. Several chemicals have been recommended and certain surgical procedures described to achieve this express purpose. Less attention has been given to preventing syngamiasis in exposed birds. Among the few contemporary reports on prophylaxis of this parasitic state are those of Lampe,[3] who demonstrated that adequate sanitation and segregation of sick birds reduces syngamiasis, Herman and Kramer,[2] who found that fumigating the ground of pheasant pens with ethylene dibromide reduced the incidence of gapeworm infections, and Girotto,[1] who used a number of chemical agents to destroy the earthworm carriers of *S. trachea*.

The work described here was done to relieve an acute persistent syngamiasis problem on a large pheasant ranch and provide an effective method of controlling similar infections in an area where domestic pheasant rearing is an expanding endeavor.

MATERIALS AND METHODS

The field trials were performed on Griffith Island, in Lake Huron, on a ranch where 6000 pheasants have been reared annually on the same premises for the past 18 years. Syngamiasis on the island antedated the trials by at least 10 years. Reported annual morbidity in the young pheasants was

* Present address: Griffith Island, North Keppel, Ontario, Canada.

variable and ranged as high as 30%. Mortality was never more than 10% in any one season.

The ranch consisted of 15 holding pens enclosed by link-wire fencing, each 50 feet square and in a single line. A brooder house was provided for each pen, and access into the pens for the birds was direct. Drainage away from the brooder houses was excellent. The ground, which was originally a beach, consisted of smooth flattened stones between which a sparse quantity of topsoil had accumulated. This soil, in all pens, supported a grass and weed cover and earthworms. These earthworms were distributed throughout the ranch and adjacent rangeland. Their numbers were greater where moisture persisted and where organic materials accumulated. In the pens, which were open to the sun and wind, they were plentiful during the spring, but less easily found during the hot season, except after a rain. No attempt was made to estimate the number of earthworms in each pen. Trees overshadowed one edge of pens 5, 6, 7, and 8 sufficiently to prevent the soil from drying out during even the hottest part of the summer. Earthworms could always be found there.

Prior to the various trials, an effort was made to find the intermediate hosts of *S. trachea*. Earthworms were the only invertebrates found to bear the larvae of *S. trachea*. Earthworms taken at random from various of the pens were examined. Those bearing encysted worm larvae were fed to susceptible uninfected pheasants. The fact that the birds all showed signs of syngamiasis and bore *S. trachea* worms when they were autopsied was accepted as proof that pheasants in the various test pens could be infected by consuming earthworms. The role that embryonated *S. trachea* eggs or that free-living larvae of the parasite might play in causing syngamiasis was not investigated.

All pheasants on the ranch were received from a single game-bird hatchery, at a distance from the island. Disease-free birds were received at three weeks of age, 400–1200 at a time. Shipments were 1–3 weeks apart, beginning in late May and ending in early August. After three weeks the birds were released from the brooder houses into the various pens,

where they remained for 5–6 weeks. Each pen contained about 200 birds at one time, and was used at least twice during a season.

Where possible, the brooder houses were left open once the birds were released into the pens, and the water and feed vessels were placed in the houses. The birds usually came into brooder houses at night, to roost or for protection from the weather, unless the houses were occupied by new lots of birds.

Since the ranch was a commercial establishment, free slaughter of birds to estimate the degree of infection was not permitted.

The incidence of syngamiasis was estimated chiefly from overt signs of disease. Counts were made of obviously affected birds in the various test pens. Fifteen minutes per pen was sufficient to register all reacting birds present. The chief signs looked for were a "cough" and periodic dyspnea. Birds on untreated ground were compared with birds placed in pens treated with the various chemicals. Permission was obtained to kill one or two affected birds per pen to confirm the presumptive diagnosis of syngamiasis. Birds that died in the pens during the test periods were collected for autopsy.

The holding pens were customarily left vacant from late August until June except for four pens used to hold a number of adult birds in October until shipment for use as breeding stock.

Seven chemicals were applied to the ground in certain holding pens to destroy earthworms. As indicated in Table 1, these were applied at various times during three successive years. In each case the ground treatments were completed at least a month before birds were released into them and regardless of when the observation of effect might be registered. The following chemicals were used.

1) *Diesel fuel oil* was applied by knapsack sprayer at 0.16 imperial gal. per sq ft of pen area. The ground was saturated about 1 in. deep.

2) *Calcium chloride* pellets were spread dry over the ground in one pen at 1.92 oz per sq ft. The deliquescent salt soon dissolved and was absorbed into the ground.

3) *Chlordane (25% wettable powder)* mixed in equal amount with methoxychlor (50% wettable powder) spread over the ground of one pen at 0.16 oz per sq ft (.02 oz chlordane, .04 oz methoxychlor). The dry powder on the ground was sprinkled twice with a garden hose to wash the insecticide off the vegetation and into the soil.

4) *Aldrin (20% emulsifiable concentrate)* was mixed in a gallon of water and sprayed evenly over the ground surface in one pen at 0.092 ml of concentrate per sq ft. The chemical was delivered with a garden hose–siphon device.

5) *Chlordane (40% wettable powder)* was applied to the ground of one pen in a liquid concentrate (by the hose-siphon) at 362 mg of powder per sq. ft.

6) *DDT (50% wettable powder)* suspended in an aqueous solution was sprayed in one pen (by hose-siphon) at 545 mg of powder per sq ft.

7.) *Mercury bichloride* was sprayed on the ground in two pens in October, 1957, at 60 mg per sq ft. One of the pens received the same again in April, 1958. In an enlarged trial 13 pens received the treatment ($HgCl_2$) in the fall of 1958, and eight of these were given a second treatment in the spring of 1959.

As a prophylactic measure, but not to destroy earthworms, phenothiazine in a 3% concentration was given in the feed continuously to 200 five-week-old pheasants from a week prior to being released into an infected pen until they were 12 weeks old. The phenothiazine was used to destroy larval *Syngamus* worms before they could migrate in the bodies of their hosts.

OBSERVATIONS

Syngamiasis occurred in 10 days at the earliest in the control pens and in pens where treatment was ineffective. This observation coincided with those made previously by the ranch operator. There was no outstanding difference between birds of one shipment as opposed to another in numbers of cases per pen or intensity of disease. The various shipments of three-week-old pheasants varied in quality from batch to batch and year to year, but the birds released into the pens were capable

of surviving to adulthood under normal conditions. Weak birds usually did not survive shipment.

Birds slaughtered always contained *S. trachea* worms. No other respiratory condition was detected to account for disease signs.

Table 1 presents the results of treatment of the various pens during three years. As indicated, calcium chloride, chlordane and methoxychlor combined, aldrin, and chlordane gave no substantial protection against syngamiasis.

Diesel oil gave full protection to the first group of birds released into the test pen after treatment. The rancher reported that the second lot showed no signs of disease while they remained in the pen. The fuel oil killed all the vegetation in the pen for most of one season. Earthworms were observed in the pen during the following year, and clinical cases were encountered.

DDT afforded protection. Diseased birds were not observed until late summer in the second lot of pheasants in the treated pen.

Birds given phenothiazine reduced their feed intake and lost weight. They did not grow normally, were visibly weak and depressed, and presented a distinct bronze or rust color in the feathers.

DISCUSSION

The numbers of infected birds and intensity of syngmiasis were obviously greater among birds released to range outside of the pens than in the pens. This accounted for the estimate, made by the ranch operator, of 30% morbidity and 10% mortality in certain seasons.

Comparison could not be made between incidence in control and test pens and incidence in previous years. No comparison was made to see if the vitality of the hatches influenced the incidence of disease.

It was possible that pheasants were infected directly by consuming embryonated eggs of *S. trachea*. How many eggs were present per square foot of pen area was not determined. Free-living larvae could also be a source of infection—but not a major one. These larvae are quite nonresistant to drying,

and the area was quite exposed to the wind and sun for much of the pheasant–rearing season. The ostensible absence of earthworms in pens where disease did not appear, and vice versa, was taken as presumptive evidence that earthworms were major carriers of *S. trachea* infection under the conditions of these trials.

Table 1. Gapeworm[4] disease in pheasants (200-bird lots) at various exposure periods in pens given various treatments.[1]

	1957[2]			1958[3]			1959[4]	
Pen no.	Spring[5] treatment	No. showing disease (after days exposed)	Fall[6] treatment	Spring[5] treatment	No. showing disease (after days exposed)	Fall[6] treatment	Spring[5] treatment	No. showing disease (after days exposed)
1			$HgCl_2$	NT	0(42)		X	13(24)
2	X	19(42)	$HgCl_2$	$HgCl_2$	0(42)	$HglCl_2$	$HgCl_2$	0(24)
3	oil	0(42)		NT	4(18)	$HgCl_2$	$HgCl_2$	0(24)
4						$HgCl_2$	$HgCl_2$	0(24)
5	P[7]	2(35)[8]		Ald	22(35)	$HgCl_2$	$HgCl_2$	0(31)
6	$CaCl_2$	5(15)				$HgCl_2$	$HgCl_2$	0(31)
7	C&M	6(15)				$HgCl_2$	$HgCl_2$	0(31)
8	X	5(15)		DDT	0(35)		$HgCl_2$	2(31)
					3(42)[9]			
9				X	21(35)		$HgCl_2$	4(31)
10							$HgCl_2$	3(37)
11				C	7(14)		$HgCl_2$	7(39)
12						$HgCl_2$	$HgCl_2$	0(39)
13						$HgCl_2$	$HgCl_2$	2(39)
							X	9(24)
15							$HgCl_2$	1(24)

[1] Treatments: X, control, NT, no treatment in spring of the test; oil, diesel oil; $CaCl_2$, calcium chloride pellets; C, chlordane; C &M, chlordane and methoxychlor; Ald, aldrin; DDT, dichloro-diphenyl-trichloroethane; $HgCl_2$, mercury bichloride; P, phenothiazine in feed.

[2] All results are for the first lot of birds of the year.

[3] All results are for the first lot of birds (except lot 2 in pen 8 also shown)

[4] All results are for the second lot of birds of the year.

[5] Spring treatments of pen area were at least 1 month before birds released into pens.

[6] Fall treatments were in October, after the birds were gone.

[7] Beginning when the birds 5 wk old, given 6 wk in feed as prophylaxis to destroy *Syngamus* larvae in host.

[8] Two died from effect of treatment.

[9] Information for lot 2, which followed lot 1 in this pen.

Diesel fuel oil was effective for a season in destroying earthworms in the ground and was instrumental in the prevention of syngamiasis in pheasants released into the pen so treated. Labor and material costs, however, make its use impractical for control of syngamiasis on commercial game farms.

Mercury bichloride is cheap and procurable, and when used twice a year in the quantities indicated, was effective in controlling syngamiasis. The material is corrosive to metallic equipment and may accumulate in the ground to a point where birds may be harmed. Single applications at the level in these trials reduced infections but did not eliminate them entirely. DDT was quite effective in controlling syngamiasis, but the protection began to fail after 12 weeks.

Phenothiazine was highly protective, but a 3% concentration in the feed was not practical, since treated pheasants were visibly starved and weak and some deaths resulted.

The experiments and the data were restricted by certain circumstances. Absolute control of environment and free handling of the subjects were not permitted in this commercial enterprise. The ranch was distant from the authors' headquarters, so working periods were limited in both number and time. Day-to-day observations were not possible.

Syngamiasis is a dramatic disease in its extreme form, and acute signs are easily observed, but lightly infected birds are sometimes difficult to detect. Thus, no assurance was forthcoming that all infections were detected during a count. Further, since syngamiasis is a progressive condition, incidence would be influenced by exposure time, which was not uniform throughout the various pens; the number of cases, especially among controls increased until birds were released. The fact that the birds were released into the pens at various periods as they matured accounts for the different exposure times noted in Table 1.

SUMMARY

Of seven chemicals used to destroy earthworms, intermediate hosts of *Syngamus trachea*, in the ground of pheasant runs, diesel fuel oil and mercury bichloride applied to the

ground twice a year were highly effective in controlling outbreaks of syngamiasis. DDT held infections to a low incidence. Phenothiazine in the feed in 3% concentration gave a high degree of protection against *S. trachea* infections in five-week-old pheasants but was definitely toxic to pheasants under 12 weeks old.

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REVACCINATION OF CHICKENS AGAINST NEWCASTLE DISEASE BY VENT, WING-WEB, AND INTRAMUSCULAR ROUTES

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LIVE virus Newcastle disease (ND) vaccine is commercially available, and still widely used, for administration by wing-web and intramuscular routes. Many use this vaccine in the expectation of achieving high immunity levels from revaccination of their replacement stock—presumably because of the relatively greater virulence of the vaccine virus employed.

This study was conducted to determine the secondary,

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or booster, immunity response arising from administering Newcastle disease virus (NDV), N-47 strain, by the vent-drop method[6] and to compare it with that elicited by Roakin strain[1] virus administered by conventional wing-web method and intramuscular injection.

MATERIALS AND METHODS

Vaccines. The N-47 strain NDV used for vent-drop administration consisted of infected allantoic fluid diluted with sterile distilled water to give between $10^{6.0}$ and $10^{6.5}$ embryo infective dose$_{50}$ (EID_{50}) per ± 0.016 ml. This was applied in *one* drop to the unopened vent of each chicken from a plastic bottle with a 19-gauge needle attached for delivery. In field trials, the NDV was combined with the viruses of infectious laryngotracheitis (LT) and infectious bronchitis (IB), and administered by vent as a triple vaccine (Triplvax, American Scientific Laboratories, Inc., Madison, Wisconsin).

Roakin strain NDV from a commercial production lot was employed for intramuscular and wing-web vaccination. The dried virus preparation was reconstituted and diluted in sterile distilled water so that each bird received between $10^{6.0}$ and $10^{6.5}$ EID_{50} with either route. When given intramuscularly, 0.5 ml was injected into the right leg. Wing-web vaccination was done with a double needle (sewing machine) applicator in the right wing.

Chickens. Straight-run broiler chicks were used in the laboratory trials, and all were vaccinated initially at five days of age when they possessed varying degrees of congenital ND antibody as measured by the serum neutralization test. This vaccination was done either through the drinking water (B-1 strain) or by vent-drop (N-47 strain). Revaccination was done at 5 and 6 weeks of age in the respective experiments. At that time the chickens were divided into their various lots and held in colony houses.

The field trials were conducted on a poultry fancier's farm where 26 different breeds of varying ages were present. Initial vaccination and revaccination, in all cases, was done with the triple vaccine. About 6 months before the trials began, 300 pullets had been vaccinated with killed NDV; at the beginning of the trials the ND immunity levels, based on

hemagglutination inhibition (HI) and serum neutralization tests, were very low or nonexistent. Hence, this flock was used as a control or an indicator of possible adventitious ND infection.

Serology. In the laboratory trials, serum samples were obtained from vaccinated and control chickens at the time of revaccination and 20 days later. In the field trials, serums were taken on the day of revaccination and 5 and 6 weeks later. A beta HI test procedure was used as described elsewhere.[5]

Challenge. GB strain[2] NDV-laden allantoic fluid was given by drop ($\pm$0.016 ml) to the eye of each bird—10^6 EID_{50} per bird. The birds were then held about 2 wk for observation. Criteria for resistance to challenge were the absence of nervous signs and/or death.

RESULTS

Table 1 summarizes data from the first trial (N-47 NDV by vent-drop compared with the Roakin strain virus by wing-web). The vaccine given by vent induced satisfactory secondary immunity response as measured serologically by the HI test; the geometric mean titer was increased at least threefold. In contrast, wing-web revaccination stimulated no rise in serological immunity. Actually, serum titers declined between primary and secondary vaccination. As might be expected in a short-term immunity experiment of this kind, none of the vaccinated or revaccinated chickens were lost from challenge.

Table 2 shows the results of the second laboratory trial. Vent-drop vaccination again caused at least a threefold increase in titer. As in the first experiment, chickens revaccinated by wing-web showed no response. Those vaccinated intramuscularly also showed no significant rise in immunity over pre-revaccination titers. In this experiment, the chicks were vaccinated initially when 5 days old by vent-drop with N-47 NDV. Immunity at revaccination was high, and those birds not revaccinated were still able to resist challenge at 9 weeks old. Even in the presence of this high immunity, however, the secondary immunity response to vent vaccination was good.

Table 3 gives the results of revaccinating two pullet flocks under field conditions. In each case, revaccination was by

vent-drop using the triple vaccine that contained N-47 NDV. Data from these trials confirm the previous laboratory findings; HI titers in each flock were raised substantially. Flock 1 was vaccinated initially at 14 days old. At revaccination (22 weeks), birds from this flock were challenged; only one of 38 showed signs of ND. The flock was coming into production at that time but revaccination did not depress or retard lay. Likewise, the challenged birds were not affected in this respect.

DISCUSSION

Previous work[6] indicates that the N-47 strain NDV can be given by vent-drop for initial vaccination, and may elicit better immunity than the B-1 strain administered by drop to the nostril or eye. Since no previous information has been presented on the booster effect of vent administration, this study was undertaken and some of the practical applications of ND revaccination were explored. No attempt was made to compare the secondary immunity response to all vaccination methods and NDV strains available, because of the extensive physical facilities required. The wing-web and intramuscular routes of administering Roakin NDV were selected because occasional ND outbreaks occur in flocks revaccinated by these procedures.

This study shows that the vent route gives a substantial boost in immunity that may not be obtained with nonrespiratory routes such as intramuscular and wing-web. The poor or negative immunity response obtained with the more virulent Roakin strain used intramuscularly or by wing-web, is not surprising in view of the work of Markham *et al.*[4] It is possible that the virus given by these two methods may on revaccination be neutralized before it has an opportunity to infect susceptible cells. Apparently this does not occur with vent administration of a strain even considerably less virulent than Roakin. So-called "breaks" in the immediate post-revaccination period may be explained from the failure to obtain an anamnestic response—as was the case in this study. Although the HI antibody response may not be a true criterion of actual bird protection, it does, nevertheless, provide a useful technique in a study of this kind. The results plus field reports lend credence to the inability of Roakin wing-web and

Table 1. The secondary immunity response from chickens revaccinated for Newcastle disease at five weeks after initial vaccination when five days old.

Lot no.	Method of revaccination	When tested[1]	HI titer distribution 0	8	16	32	64	128	256[2]	Geo. mean	Challenge results[3]
1	Vent-drop	R	4	2	5	3		1	4	20	—
	(N-47 strain)	20	1		3	1	4	2	7	73	0/18
2	Wing-web	R	3	1	3	3	2	2	6	37	—
	(Roakin strain)	20	6	2	7	4	1			8	0/20
3	Controls	R	1	1	1	1	2	2	2	42	—
	(not revaccinated)	20	5	2	2		1			2	0/9[4]
4	Controls	R	5							0	—
	(susceptible)	20	5							0	4/4[4]

[1] R, at revaccination; 20, 20 days later. [2] Highest serum dilution tested. [3] Number with signs/number challenged. [4] One bird lost from each when bled.

Table 2. The secondary immunity response from chickens revaccinated for Newcastle disease at six weeks of age after initial vaccination when five days old.

Lot no.	Method of revaccination	When tested[1]	HI titer distribution 0	8	16	32	64	128	256[2]	Geo. mean	Challenge results[3]
1	Vent-drop	R		1	2	3	8	4	4	64	—
	(N-47 strain)	20					1	4	17	208	0/22
2	Wing-web	R		2	6	5	5	3	1	32	—
	(Roakin strain)	20	1	4	6	8	1	2	1	23	0/23
3	Intramuscular	R			2	6	6	3	3	64	—
	(Roakin strain)	20			1	3	8	3	5	80	0/20
4	Controls	R				3	3	1	2	73	—
	(not revaccinated)	20	2	2	3			1		9	0/9[4]
5	Controls	R	9							0	—
	(susceptible)	20	9							0	8/9

[1] R, at revaccination; 20, 20 days later. [2] Highest serum dilution tested. [3] Number with signs/number challenged. [4] Discrepancy in numbers tested and numbers challenged is the result of insufficient serum.

[1] R, at revaccination; 20, 20 days later. [2] Highest serum dilution tested. [3] Number with signs/number challenged. [4] Discrepancy in numbers tested and numbers challenged is the result of insufficient serum.

Table 3. The secondary immunity response from chickens revaccinated for Newcastle disease under field conditions with N-47 strain virus administered by drop to the vent in combination with the viruses of infectious bronchitis and infectious laryngotracheitis.

Flock no.	Flock history	When tested[1]	HI titer distribution 0	8	16	32	64	128	256[2]	Geo. mean	Challenge results[3]
1	1300 vac. at 14 days old; revac. at 22 wk.	R	4	3	1	1				6	1/38
		42				2	1	1	11	169	—
	Controls	R	3							0	6/9
		42	3							0	—
2	1100 vac. at 14 days old; revac. at 13 wk.	R	4	1	3					4	—
		35					2	6	7	158	—
	Controls	R	4							0	—
		35	3							0	—

[1] R, at revaccination; numbers are days after revaccination. [2] Highest serum dilution tested. [3] Number with signs/number challenged.

intramuscular vaccine in stimulating a significant rise in immunity.

Cole and Hutt[3] observed differences between strains of White Leghorns in the morbidity and mortality that occurred following wing-web vaccination. This work, perhaps, is of significance in interpreting some of the clinical effects from wing-web or intramuscular vaccination often seen under field conditions. Paralytic signs are sometimes manifested shortly after revaccination; it is possible, in these cases, that the residual immunity from primary vaccination has disappeared and the virus is able to exert itself in full virulence and reflect its neuropathogenicity. At times this reaction will assume the proportion of a typical ND outbreak. Such problems are not only vexing to the poultryman but the vaccine manufacturer as well.

SUMMARY

The secondary immunity response following revaccination of chickens for Newcastle disease (ND) was studied and the results given. Revaccination with N-47 strain Newcastle disease virus (NDV) by the vent-drop method boosted immunity levels substantially when measured serologically, irrespective of whether the vaccine was given singly or in combination with the viruses of infectious bronchitis and infectious laryngotracheitis. Roakin strain NDV given intramuscularly or by wing-web failed to stimulate a significant increase in ND hemagglutination-inhibition antibody titers. Problems arising from wing-web and intramuscular vaccination are discussed.

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EFFECT OF TEREPHTHALIC ACID ON THE ACTIVITY OF CHLORTETRACYCLINE AND OXYTETRACYCLINE*

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DIETARY efficacy of the tetracycline antibiotics is significantly enhanced by terephthalic acid (TPA) in the ration.[1-6] The dietary quantity of chlortetracycline (CTC) can be reduced fourfold by 0.5% TPA.[2,6]

In view of apparent differences in biological efficacy among the tetracycline antibiotics given orally, interest remains high in the effect that TPA has on CTC and oxytetracycline (OTC).[7,9] To gain some conclusive comparative data on the extent to which TPA potentiates these antibiotics, CTC and OTC were orally administered simultaneously with TPA to chicks inoculated with infectious synovitis agent.

EXPERIMENTAL

Fifteen unsexed White Rock–Vantress chicks were used for each treatment. These were maintained in electrically heated batteries equipped with wire floors and located in a constant-temperature controlled-humidity room. The basal ration was a commercial broiler ration containing 3 g of penicillin per ton, 0.005% 3-nitro-4-hydroxyphenylarsonic acid,

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and 0.0125% nicarbazin. Separate treatments were maintained in which crystalline hydrochloride and feed-grade forms of the antibiotics were compared. TPA (technical grade) was added to certain treatments at the rate of 0.5%. Both the antibiotics and the TPA were added to the basal ration when the chicks were one week old, and given continuously for the duration of the experiment.

Except in the uninfected control treatments, each of the 15 birds per treatment was experimentally infected via the foot pad when two weeks old by injecting 0.25 ml of a virulent embryo-grown culture of infectious synovitis agent 1853. Uninfected controls were maintained on the basal ration and the basal ration plus 0.5% TPA. The birds were observed and the mortality recorded daily. The severity of infection was determined as previously outlined by Shelton *et al.*,[8] and recorded weekly during the 4-week experimental period.

RESULTS

Table 1 summarizes the effects of the infectious synovitis agent 1853 on unmedicated birds. During the 4-week experimental period all birds became severely affected. The degree of swelling was maximal, and the spread of the infection occurred in all birds. Mortality was 20–40%. Weight

Table 1. The response of control treatments to terephthalic acid and the infectious synovitis agent 1853.

Severity of infection[1]	Uninoculated		Inoculated	
	No TPA	0.5% TPA	No TPA	0.5% TPA
No. birds infected	0	0	15	15
Degree of swelling	0	0	9.0	9.0
Extension	0	0	15	15
Mortality	0	0	3	6
Weight gain (g)	639	639	139	162
Feed conversion	2.10	2.08	4.33	6.55

[1] Severity of infection shown by the following: *Number* of birds developing disease; *degree* of swelling, based on a scoring system of 0 to 9, applies only to leg of inoculation; *extension*, number of birds in which infection has spread to joints other than those of the inoculated leg.

gain was about 25% of that of the uninfected controls, and feed conversion was very poor. TPA in the absence of antibiotics did not significantly alter the course of the disease. No signs of the disease appeared in the uninoculated birds. TPA did not influence the weight gain or feed conversion of the uninoculated birds.

Table 2 summarizes the influence of TPA on CTC and OTC treatments. Analysis of variance was made to determine significance of results. The form of the antibiotic (crystalline hydrochloride or feed grade) had no significant ($P>.05$) effects. As a result, these treatments are considered as replicates; however, to illustrate the relatively minor variation that occurred, the treatments are shown separately in Table 2.

Regardless of the form in which the antibiotics were provided, neither 12.5 nor 25 g of CTC or OTC in the ration was effective in protecting the birds from developing synovitis. The number of infected birds decreased significantly when 50 g of CTC was added, but 100 g of OTC was needed for a similar effect. In the presence of 0.5% TPA, however, even 12.5 of CTC reduced the number of birds infected by 37%. The effect was not significantly different from that produced by: 1) 50 g of CTC, 2) 100 g of OTC, or 3) 50 g of OTC plus 0.5% TPA. The potentiation response of 0.5% TPA on 12.5 g of OTC was not detectable under these conditions.

At 25 g per ton plus TPA, CTC controlled the infection so that no signs were apparent in 90% of the birds. This was comparable to the response to: 1) 100 g of CTC or 2) 100 g of OTC plus 0.5% TPA. Only a slight potentiation effect by the TPA was apparent at the 25-g concentration of OTC: only 10% of the birds were protected. Protection was about 50% from 50 g of OTC plus TPA, and about 100% from 100 g of OTC plus TPA.

A more finite evaluation was possible by observing the average degree of swelling. Here again, both antibiotic forms (crystalline and feed grade) were considered together. In birds given 12.5 g of CTC and OTC, the respective average degrees of swelling were 6.1 and 7.0, slightly less than the 9.0 for the infected controls. This amount of antibiotics was beneficial in

controlling spread as a generalized infection of only 37 and 57%, respectively, in contrast to 100% for the infected controls. As the concentrations of these antibiotics were increased to 25, 50, and 100 g per ton of feed, the average degree of

Table 2. The biological response of chlortetracycline and oxytetracycline in suppressing the effects of infectious synovitis agent 1853 as influenced by terephthalic acid.

Amount of antibiotic g/ton	Crystalline				Feed-grade			
	No TPA		0.5% TPA		No TPA		0.5% TPA	
	CTC	OTC	CTC	OTC	CTC	OTC	CTC	OTC
No. birds infected[1]								
12.5	15	15	10	15	13	15	9	15
25	15	15	3	13	12	15	0	14
50	7	14	0	7	6	14	0	9
100	2	8	0	0	1	8	0	1
Degree of swelling[1]								
12.5	7.7	6.3	2.1	6.6	4.4	7.7	1.9	6.9
25	5.3	5.3	0.2	4.2	2.5	6.9	0	4.4
50	1.8	4.5	0	1.5	1.0	1.3	0	1.3
100	0.5	1.6	0	0	0.3	1.3	0	0.2
Extension [1]								
12.5	10	6	1	11	1	11	0	6
25	5	5	0	2	0	8	0	4
50	1	1	0	0	0	3	0	0
100	0	0	0	0	0	0	0	0
Mortality								
12.5	0	1	1	1	0	4	0	0
25	1	3	0	0	0	3	0	2
50	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0
Weight gain (g)								
12.5	399	419	631	396	438	384	618	481
25	576	522	637	591	624	365	640	599
50	602	603	728	627	656	559	635	669
100	614	637	696	644	693	661	656	633
Feed conversion								
12.5	2.33	2.35	2.20	2.69	2.32	3.49	2.14	2.20
25	2.39	2.21	2.07	2.18	2.10	2.84	2.11	2.32
50	2.11	2.12	2.01	2.17	2.05	2.20	2.09	2.04
100	2.06	2.03	2.06	2.03	1.98	2.02	2.08	2.22

[1] Severity of infection shown by the following: *Number* of birds developing disease; *degree* of swelling, based on a scoring system of 0 to 9, applies only to leg of inoculation; *extension*, number birds in which infection has spread to joints other than those of the inoculated leg.

swelling decreased to 3.9, 1.4, and 0.4 for CTC, and 6.1, 4.4, and 1.5 for OTC. The addition of 0.5% TPA so enhanced the efficacy of 12.5 g of CTC that the average degree of swelling was only 2.0. This response was not significantly different from that produced by: 1) 50 g of CTC, 2) 100 g of OTC, or 3) 50 g of OTC plus TPA. It took 25 g of OTC plus TPA to reduce swellings significantly. Swelling was detectable only in birds receiving 25 g of CTC and TPA; none occurred with higher amounts. With 50 g of OTC plus TPA, average swelling (1.4) was just slightly less than the 2.0 given by the 12.5 g of CTC plus TPA treatment. Equivalent effective concentrations when potentiated with 0.5% TPA were 25 and 100 g, respectively, for CTC and OTC.

Mortality was too low to be useful in evaluating the antibiotic effects.

No significant difference was apparent in weight gains between the antibiotic treatments of 12.5 g except where TPA was fed. The 12.5-g concentration of CTC and OTC increased weight gain by threefold over infected controls (418 and 391 vs. 139 g), though weight was still only two-thirds of that of the normal control (639 g). Likewise, feed conversion was depressed, though not seriously except where birds were so severely affected that they became emaciated at the terminal stages of the disease. In all treatments, variations in weight gains were closely correlated with systemic infection. Whenever antibiotic concentrations were sufficient to localize the infection, weight gains and feed conversion were nearly normal.

DISCUSSION

For sometime the interpretation of results in studies with synovitis have perplexed the authors when weight gains and feed conversions are relied upon as true expressions of the disease status of infected birds. For instance, of the antibiotic treatments in this study, significant depressions in weight gains occurred only at one concentration of CTC (12.5 g) and at two concentrations of OTC (12.5 and 25 g). When potentiated with 0.5% of TPA, the 12.5-g concentration of OTC was

the only antibiotic treatment at which a significant depression in weight gain was observed. Whenever antibiotic concentrations were sufficient to localize the infection, weight gains and feed conversion were nearly normal. Consequently, when investigators use 100–200 g of OTC per ton of feed and compare their efficacies by consideration of weight gains and feed conversions, there is little wonder that differences are not observed. On the other hand, when the development of lesions are considered critically at lower concentrations, marked differences in efficacy are detectable.

In this study chlortetracycline was about twice as effective as oxytetracycline. Twenty-five grams of CTC was as effective as: 1) 100 g of CTC or 2) 100 g of OTC plus 0.5% of TPA. In effect, then, the 0.5% TPA potentiates the CTC four times. The 100 g concentration of OTC is equivalent in efficacy to 50 g of OTC plus 0.5% TPA. Thus, the TPA potentiates the OTC two times. By comparison, the 0.5% TPA potentiates CTC twice as much as it does OTC.

SUMMARY

Twelve groups of 15 birds each were used to study the effects of terephthalic acid on the activity of chlortetracycline and oxytetracycline. The chicks were placed on the treated feeds one week prior to inoculation. Results of the various treatments during a 4-week period were based on signs of the disease, weight gain, and feed efficiency, and the degree of swelling following footpad inoculation of 2-week-old chicks with the synovitis agent. Chlortetracycline was found to be twice as effective as oxytetracycline. Five-tenths percent terephthalic acid potentiated chlortetracycline 4 times and oxytetracycline 2 times. Weight gains and feed conversion data were essentially normal where the treatment prevented systemic infection.

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ORNITHOSIS IN TURKEYS IN ALBERTA

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SEVERAL workers have reported ornithosis infection in turkeys caused by agents of *Miyagawanella* genus (psittacosis–lymphogranuloma venereum group) of low virulence, characterized by caseofibrinous exudates on the serous

membranes of the abdominal and thoracic cavities, with low mortality.[1,2,5,6] This is at variance with the mortality reported in Texas and Oregon.[3,7]

This report describes ornithosis of turkeys in Alberta, resulting from infection with an organism of apparently low virulence; successful transmission of the agent to turkey poults; and a limited serological survey of the extent of the infection in local turkey-breeding flocks.

HISTORY AND DIAGNOSIS

Flock J. During January and February, 1960, five (4 live, 1 dead) Broad Breasted Bronze (BBB) and two live White Holland (WH) turkey hens were received at the laboratory from a flock of 280, as part of a routine of submitting all dead, sick or cull birds for diagnosis. No sudden onset of disease was reported, climatic conditions for turkey raising were the most favorable for many years, and management of the flock was of the highest caliber. All five of the BBB birds and one of the WH turkeys had bacteriologically negative purulent pericarditis and aerosacculitis, suggestive of ornithosis infection. The other WH hen showed no suspicious lesions except for a kerato-conjunctivitis causing blindness in both eyes. Portions of spleen and paricardium of one of the BBB birds were ground in sterile sand and nutrient broth, and inoculated intracranially in 0.03-ml doses into six mice, all of which died between the 4th and 6th days posinoculation. Autopsy showed an excessive slightly blood-tinged peritoneal fluid and edema of the abdominal wall. Meninges were not examined. Giemsa-stained impression smears revealed bodies similar to the elementary bodies of ornithosis. Attempts to infect mice by peritoneal injection of 0.5 ml of this material were unsuccessful. Material from the WH hen showing lesions of ornithosis, inoculated intracranially into mice, caused death in 13 days, and stained smears of the mouse spleens revealed typical elementary bodies.

On January 18, 1960, nineteen birds from this flock were tested by the indirect complement-fixation test, and the entire flock was tested on April 21. The results are in Table 2.

Flock K. On February 16, two live Broad Breasted Bronze hens, from a flock of 2200, were submitted as being lethargic but thought to be individual cases. This flock, seven miles from Flock J, had comparable management conditions. Both birds showed purulent pericarditis and aerosacculitis, which proved to be bacteriologically sterile. Portions of spleen and pericardium were prepared and inoculated intracranially into four mice. All died about 18 days postinoculation, and stained impression smears revealed elementary bodies in profusion. On April 1, blood sera from 30 birds in this flock were tested for ornithosis, and on May 24 two hundred more birds were subjected to the test. The results are summarized in Table 2.

EXPERIMENTAL TRANSMISSION

Thirty-eight newly hatched Broad White poults, and sixteen White Leghorn chicks were obtained for experimental inoculation. The procedure of this experiment is summarized in Table 1.

Group A. Three poults and three chicks died from overcrowding in the first four days postinoculation. On the 9th and 15th days, one poult died, and at autopsy each revealed marked purulent pericarditis-aerosacculitis lesions. Spleen tissue from the bird that died on the 9th day killed mice about 4 days after intracranial inoculation. On the 57th day, one

Table 1. Experimental transmission.

Group	No. birds	Route	Dose	Inoculum	Transmission[1] results
A	8 poults	IP	0.25 cc	3rd passage	3/5
	4 chicks			mouse tissue	0/1
B	8 poults	Cleft	1 drop	3rd passage	2/8
	4 chicks	palate		mouse tissue	0/4
C	8 poults	IP	.25 cc	Normal mouse	0/3
	4 chicks			tissue	0/4
D	14 poults	Nil	Nil	Nil	0/12
	4 chicks				0/4

[1] Positive ornithosis on isolation or ICF/Birds tested.

bird developed perosis and was sacrificed. Lesions suspicious of ornithosis, involving the pericardial sacs and air sacs, were observed. Material from this poult, however, did not cause fatalities in mice. Serum from this bird proved negative on the indirect complement-fixation test for ornithosis.

Group B. On the 57th day postinoculation, one bird from from this group developed perosis and was sacrificed. Lesions suggestive of ornithosis were observed. Spleen and pericardial tissues from this specimen killed two mice 3 days after intracranial inoculation. Tissues and serum from this bird, submitted to the Animal Diseases Research Institute, at Hull, Quebec, were isolated and identified as an agent considered to be of the *Miyagawanella* genus. Indirect complement-fixation for ornithosis revealed a titer of 1:40.

Group C. Five poults died of overcrowding on the second day.

Group D. One poult died of injury on the 6th day.

Chicks. All chicks in the trial were sacrificed on the 49th day postinoculation, and at autopsy a number showed aerosacculitis. The mouse-lethal agent was not demonstrated from their tissues.

The remaining turkeys in the experimental group were tested by indirect complement-fixation test on the 66th day, and again on the 112th day postinoculation. The results are summarized at the bottom of Table 2. Only birds from Groups A and B (Table 1) reacted to the test with positive titers. No serological reaction was observed in Groups C and D, although the birds were housed together continuously from inoculation.

BREEDING FLOCK SURVEY

Blood was collected from Flocks J and K on the farm, and again at evisceration (Table 2). In addition, five other turkey breeding flocks were sampled at slaughter. Results are summarized in Table 2. Pericardial sacs, air sacs, and spleen showing lesions were harvested for examination. The agent of ornithosis was not recovered from any of the tissues.

Table 2. Ornithosis indirect complement fixation tests.[1]

Flock	No. birds		No. of birds yielding various titers:						
	In flock	Tested	Neg.	1:10	1:20	1:40	1:80	1:160	1:320
J	280	19	13	1		2	1		2
		276	204	26	23	9	7	3	4
K	2200	30	24	2	2	1	1		
		200	135	50	12	3			
L	2100	49	40	3	2	1	3		
M	1600	49	41	7	1				
N	220	50	49	1					
O	240	35	28	4	2	1			
P	2200	74	41	15	9	8	1		
Exptl.	25	24(66 days)	19	2	1	1		1	
		24(112 days)	19	1	2		1	1	

[1] Conducted by the Animal Diseases Research Institute, Hull, Quebec, which gave the following interpretation: Positive, 1:40 or over; Questionable, 1:20; Negative, 1:10 or lower.

DISCUSSION

Page[3] reported that the pathogenicity of turkey ornithosis agents can vary from high to low, as indicated by their lethal effects on mice on intraperitoneal inoculation. Gale[4] described an ornithosis agent of very low virulence that failed to produce clinical manifestation of the disease in unexposed contact birds. He was unable to isolate the virus until 20 days after exposure, and complement-fixation titers in contact birds remained low.

It is apparent that during the 1959 breeding season, ornithosis was present in at least two Alberta turkey breeding flocks. The agent appeared to be of low virulence:

1) Mortality was low in infected flocks, the highest totaling less than 2.5% throughout the breeding season (Flock J).

2) The percentage of birds showing serological titers of 1:40 or over to the ICF test was relatively low, the highest being under 25% even on the first sampling of infected Flock J.

3) Contact controls did not become infected from artificially infected poults, as judged by the serological test.

4) Mortality was less than 25% even in artificially infected poults.

5) The agent did not kill mice upon intraperitoneal inoculation.

Pericarditis and aerosacculitis of undetermined etiology have been found before in Alberta turkey breeding flocks. It is conceivable that some of those cases were ornithosis.

SUMMARY

Ornithosis in two turkey flocks was confirmed by serological testing and isolation of the agent. The disease was transmitted to day-old poults, but not to day-old chicks. Random blood sampling revealed titers of 1:40 or higher to the ICF test in 3 of 5 other flocks. The possible low virulence of the agent is discussed.

ACKNOWLEDGMENTS

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preting serological tests, is acknowledged. The cooperation of the Inspectors at the poultry processing plants is appreciated.

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STUDIES OF AN INACTIVATED *HEMOPHILUS GALLINARUM* VACCINE FOR IMMUNIZATION OF CHICKENS AGAINST INFECTIOUS CORYZA

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INFECTIOUS coryza has long been a major disease problem among chicken flocks in California. In the southern portion of the state, economic losses from this disease and its complications have been severe.

Vigorous attempts have been made to control the disease through such methods as isolation rearing, yearly disposal of laying flocks, and, in many instances, complete depopulation.

Such efforts have been of help, but in most cases the disease has inevitably returned.

It has been observed that chickens that have undergone an attack of infectious coryza develop a resistance or immunity, so the disease rarely occurs after infected growing birds reach maturity. This observation has led to the belief that a controlled infection with a live virulent culture of *Hemophilus gallinarum* administered at the proper time would produce good immunity with a minimum of losses from complicating diseases. Olsen,[6] Urban,[7] and Clark[3] have reduced losses from infectious coryza by inoculating living chicken-embryo-propagated cultures of *H. gallinarum* intramuscularly into uninfected replacement pullets. A study of the literature has failed to reveal any previously recorded successful vaccination against infectious coryza.

This paper reports the results of trials with a formalinized chicken-embryo *H. gallinarum* vaccine.

MATERIALS AND METHODS

Origin and maintenance of culture. Two highly virulent biochemically different strains of *H. gallinarum* were selected for seed cultures. These bacteria were recovered from the sinuses of acutely infected chickens from two widely separated ranches.

The original isolations were made on blood agar plates prepared from trypticase soy blood agar base (BBL) and 5% defibrinated rabbit blood and incubated in a candle jar. Characteristic colonies were picked and suspended in about 3 ml of infusion broth (BBL). This broth was inoculated in 0.2-ml portions into the yolk sacs of each of several seven-day-old chicken embryos. The inoculated eggs were incubated 24 hours at 37°C. Embryonic fluids including yolk and allantoic and ammiotic fluids were aseptically harvested and deposited in sterile glass tubes. Material from each tube was cultured again on blood agar plates, and the tubes of egg material were stored in a refrigerator at 4°C. This infected egg material, if found pure, was used as the seed for inoculation of fertile eggs for the vaccine. *H. gallinarum* was con-

firmed from the description in Bergey[2] and the methods described by Bornstein *et al.*[1]

Biochemical differentiation of the strains was made by the use of infusion broth containing 10% chicken serum, 1% carbohydrate, and 1% Andrade indicator. All cultures were incubated for 48 hr under reduced oxygen tension in candle jars at 37°C. The carbohydrates used were lactose, dextrose, sucrose, maltose, mannitol, dulcitol, salicin, trehalose, mannose, xylose, arabinose, dextrin, and levulose. One strain, which we identified as R-1, fermented the following carbohydrates: dextrose, sucrose, maltose, mannose, levulose, and dextrin. The other strain, S-1, did not ferment maltose. Otherwise, it produced the same carbohydrate reactions. Neither strain produced indole, and both reduced nitrates to nitrites (BBL indole nitrate medium) in 24 hours.

At the time of these trials no satisfactory techniques were established for titration or counting of bacteria within the egg fluids, and the only constant criterion of bacterial concentration was 100% embryo mortality, which occurred consistently within 12 hours.

Vaccine. Equal numbers of seven-day-old chicken embryos were inoculated via the yolk sac with each of the two strains of egg-propagated seed culture. The embryos uniformly died within 12 hours, but were held in the incubator for 24 hours before harvest. The embryonic fluids, consisting of yolk, allantoic, and amniotic fluids, were harvested aseptically, maintaining the two strains in separate containers.

Immediately after harvest and before formalinization, bacteriological cultures were made. The egg material was then formalinized to a final concentration of 0.25%, and refrigerated at 4°C. The formalinized egg material was recultured after a 48-hour holding period to determine the status of sterility.

The culture procedure was as follows: One loopfull of material was streaked on a blood agar plate and incubated in a candle jar, one loopful of material was suspended in 5 ml of infusion broth (BBL), and another loopful was inoculated into 10 ml of thioglycollate medium (BBL). Be-

cause of excess turbidity from suspended egg material in the liquid media, subcultures were made at 24 hours and reincubated to determine bacterial growth. All cultures were incubated 72 hours at 37°C, and each was examined daily for evidence of contamination or growth. The only growth expected at the purity or sterility examination was *H. gallinarum* on blood plates from cultures made before formalinization. Any other growth present was considered a contaminant. If such contamination was found the egg material was discarded as unfit for processing.

An oil emulsion type of vaccine was prepared with using the method described by Heddleston and Reisinger.[5] The technique and materials used were identical to those described with one exception: formalinized embryo-propagated *H. gallinarum* culture was substituted for artificial-media-grown formalinized suspension of *Pasteurella multocida.*

Sterility tests were made with the finished vaccine as previously described except that the cultures were incubated for 14 days. Additional biological safety tests were made as follows: Six chicken embryos 7 days old were inoculated via yolk sac with 0.1 ml of vaccine. An additional six embryos 9 days old were inoculated into the allantoic cavity with 0.1 ml of vaccine. The embryos were incubated seven days at 37°C and candled daily. Those that died were immediately cultured for bacterial contaminants. Embryo deaths within 24 hours that were proved not to be due to bacterial contamination were assumed to be nonspecific. If embryo deaths occurred after 24 hours and bacteriological cultures proved negative, further investigation was made of the presence or absence of avian viruses. Twelve healthy unvaccinated White Leghorn cockerels 3–5 weeks old were each inoculated with 1 ml of vaccine in the muscles of the leg. These birds were maintained in an isolation room for 14 days and observed daily for ill effects. They were then necropsied and completely examined for any abnormality that might be attributed to the vaccine.

Laboratory immunity trials. White Leghorn cockerel chicks raised in isolation were used in a preliminary labora-

tory immunity study. When three weeks old these birds were each identified by numbered wing bands. At the same time the chicks were individually bled for Newcastle disease hemagglutination-inhibition (HI) tests, infectious bronchitis serum neutralization (SN) tests, and pleuropneumonia-like-organism (PPLO) plate agglutination tests.

At four weeks of age, 100 of the birds, all found negative to the above serological tests, were placed in an isolation room in a sterilized Jamesway deck-type brooder. One half of the chicks were given inactivated infectious coryza vaccine in a dose of 0.5 ml, inoculated into the muscles of the thigh with a Cornwall-type multiple-injection syringe. These vaccinated chicks were identified by an additional wing band and maintained throughout the trial in the brooder with the 50 unvaccinated birds.

Two weeks later (at age six weeks) the 100 cockerels, which appeared normal and in good health, were inoculated by way of the palatine cleft with 0.3 ml of undiluted embryonic fluid infected with *H. gallinarum*. The challenge strain of *H. gallinarum* used in this experiment was isolated from a poultry farm where an especially severe form of infectious coryza existed. This strain, which we identify as R-2, can, in 24–48 hr, produce severe facial swelling in chickens inoculated intranasally or into the palatine cleft. The chicks were individually examined every 24 hours following challenge, and records kept by band number as to signs of infectious coryza.

Field trials. Nine separate field trials with the vaccine involved 28,308 vaccinated and 19,665 unvaccinated controls. The first six trials were conducted on one of the breeding facilities of Demler Farms. For genetic experimental purposes, this flock has been intentionally maintained infected with chronic respiratory disease (CRD), infectious coryza, and pasteurellosis. The *Pasteurella* infection experimentally maintained was of an atypical type that is very common in southern California. The causal agent was described by Clark and Godfrey[4] as *Pasteurella* new species. It has been a repeated experience that healthy chicks on this farm be-

come sick within two weeks of being placed 100 feet or nearer to the infected birds. Inevitably, the disease complex begins with infectious coryza, which then becomes complicated by CRD and pasteurellosis. Mortality and cull-outs from this disease complex are usually about 20% by the time the pullets are of laying age. The seventh, eighth, and ninth field trials were conducted on commercial egg-producing ranches.

All of the chickens involved in the nine trials were White Leghorn and White Leghorn cross pullets. All flocks had similar disease problems, beginning with infectious coryza and complicated by CRD or CRD plus pasteurellosis. All nine trials included a similar virus-vaccination program: fowl pox, 1-5 days; infectious bronchitis, 10 days and 10 weeks; Newcastle disease, 4 weeks and 14 weeks; laryngotracheitis, 7–9 weeks.

The dose of *H. gallinarum* vaccine used in all field trials was 0.5 ml. The product was inoculated intramuscularly at ages varying between 3 and 6 weeks. Time of vaccination was adjusted according to the usual history of exposure and occurrence of infectious coryza on each ranch. In all field trials the birds were vaccinated no less than two weeks or more than eight weeks before the expected outbreak of infectious coryza on the ranch involved. In eight out of the nine trials, birds were brooded in isolated brooder houses with little disease exposure before 6 weeks of age. In trial no. 9 the vaccine was administered at an earlier age since that ranch occasionally had outbreaks of infectious coryza in the brooder house.

In all trials chickens receiving the vaccine and unvaccinated controls of the same age were brooded and grown together under identical conditions. In trial no. 8 the chicks were brooded on the floor until 6 weeks old, and then placed on wire in growing pens; in the other trials the chicks were brooded on wire in hot-water-type brooders and at 6–7 weeks old moved to wire-floor multiple-bird growing cages. The latter method is the practice most common in this area. The control birds and vaccinated birds were placed in adjoining

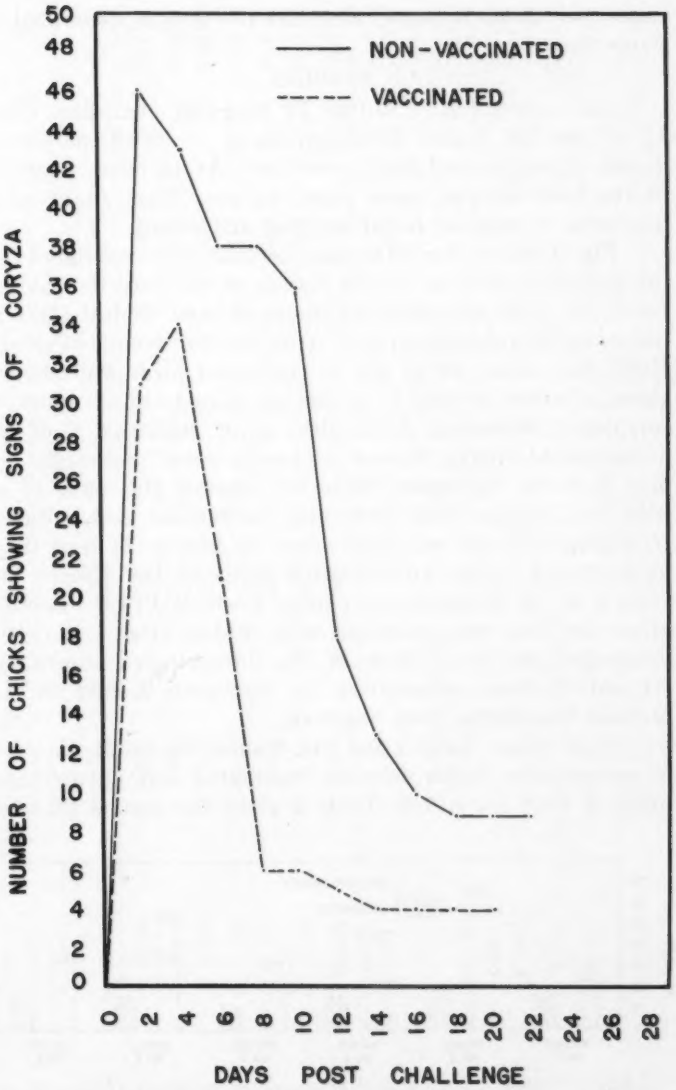

Fig. 1. Comparison of effects of artificially induced infectious coryza on vaccinated vs. unvaccinated chicks.

cages, and in no instance, were the two groups separated by more than four feet.

RESULTS

Laboratory trial. Within 24 hours of challenge, about 1/4 of the 100 chicks developed signs of infectious coryza (nasal discharge and facial swelling). At 48 hours about 3/4 of the birds showed some signs, varying from slight nasal discharge to massive facial swelling and edema.

Fig. 1 shows the difference between the vaccinated and unvaccinated birds as to the course of the infection. At 48 hours, 46 of 50 unvaccinated chicks showed clinical signs of infectious coryza, compared to 31 of the 50 vaccinated chicks. Eight days later, 38 of the unvaccinated birds still showed signs, whereas all but 6 of the vaccinated chicks appeared completely recovered. At 21 days after challenge, 9 of the unvaccinated chicks showed a chronic nasal discharge, and only 3 of the vaccinated birds still showed the signs of an infection. All the birds were bled, necropsied, and cultured. *H. gallinarum* was recovered from the sinuses of 5 of the 9 unvaccinated chicks still showing signs of the disease and from 2 of the 3 vaccinated chicks. Positive PPLO agglutination reactions were obtained with serums from two of the vaccinated and from three of the unvaccinated chicks. All HI and SN tests, respectively for Newcastle disease and infectious bronchitis, were negative.

Field trials. Table 1 and Fig. 2 show the results in terms of comparative losses between vaccinated and unvaccinated birds in field trials 1–6. Table 2 gives the results of trials

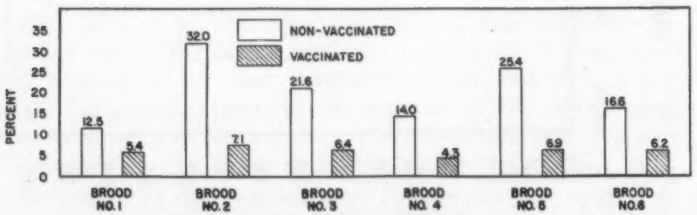

Fig. 2. Comparison of percent mortality between chickens receiving inactivated infectious coryza vaccine and those not vaccinated.

Table 1. Results of first six field trials with birds 4½–20 weeks old.

Brood No.	Vaccinated			Unvaccinated controls		
	No. at 4½ wk.	No. dead or culled by 20 wk.	% loss	No. at 4½ wk.	No. dead or culled by 20 wk.	% loss
1	4,198	227	5.4	2,312	289	12.5
2	3,452	245	7.1	2,001	640	32.0
3	3,787	242	6.4	1,891	409	21.6
4	4,136	178	4.3	2,948	413	14.0
5	3,705	256	6.9	2,202	559	25.4
6	3,380	210	6.2	1,741	289	16.6
Total	22,658	1,358	Av. 6.1	13,095	2,599	Av. 20.4

Table 2. Results of field trials 7, 8, and 9.

Experimental ranch	Age at vaccination	Age at final tabulation	Vaccinated birds			Unvaccinated controls		
			No.	Dead or culled	% loss	No.	Dead or culled	% loss
7	6 wk	10 wk	1,050	42	4.0	3,450	1011	29.3
8	4 wk	12 wk	3,000	101	3.4	3,000	302	10.6
9	3 wk	9 wk	1,600	5	0.3	120	5	4.2

7–9. Results are incomplete for the last two field trials, which were still in process as this paper was being prepared. The mortality records include losses from all causes.

DISCUSSION

In all nine field trials a natural outbreak of infectious coryza occurred at the expected time: 1–6 weeks after the chicks were moved to the growing houses. In all instances both vaccinated and unvaccinated pullets developed some signs of the disease. As infection progressed, the difference between vaccinated and control groups was outstanding. Some vaccinated pullets showed signs of infectious coryza (facial swelling and nasal discharge), but there was very little observable loss of appetite, and recovery was usually rapid. Unvaccinated birds, in contrast, displayed progressively more obvious symptoms of infectious coryza, with development of the usually expected complicating secondary infections. A certain percentage of the losses from mortality and cull-outs can be attributed to other diseases and environmental factors (assumed to be proportionally equal among vaccinated and unvaccinated groups).

The trials indicate that the inactivated infectious coryza vaccine bestowed a fair degree of protection. In no instance was the disease completely prevented, but the resistance developed appeared to be enough to help prevent complicating respiratory diseases.

Trials are being conducted to investigate the length of immunity conferred by a single injection of the vaccine compared with two injections at various intervals. Studies are also being made of methods of standardizing the vaccine through development of techniques involving titration as well as serotyping and biochemical differentiation of strains.

SUMMARY

The results are reported of trials with a chicken-embryo-propagated formalin-inactivated mineral-oil-emulsified *Hemophilus gallinarum* vaccine. Experiments reported cover a preliminary laboratory trial, and 9 field trials involving 28,308 vaccinated and 19,665 unvaccinated controls. In all

trials, damaging effects from exposure to infectious coryza were less among the vaccinated groups than the controls. Although vaccinated chickens developed signs of infectious coryza (facial swelling and nasal discharge) after challenge, recovery was early and with few complications. Control birds, in contrast, developed more severe signs of the disease, with severe malaise and anorexia, followed by complications of CRD and pasteurellosis.

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Helly's, Schaudinn's, and Zenker's fluids. Sections were cut at 6 and 3 μ and stained in Giemsa, hematoxylin and eosin, Heidenhain's hematoxylin, PAS, and Gomori trichrome, and by the method of Tomlinson and Grocott. Gelfoam blocks were soaked in exudate from the lesions, and these were fixed in 10% formaldehyde, sectioned, and stained as above. Occasionally the oropharyngeal mucosa was dissected from the bony structures, pinned on cardboard, and fixed prior to sectioning. Comparable studies were made on flagellate-free nonimmune pigeons as controls.

RESULTS

The trichomonads were recognizable on the direct smears, regardless of the method of fixing and staining. Schaudinn's fixation followed by Heidenhain's hematoxylin staining gave the best results, and Giemsa and hematoxylin and eosin the poorest.

The standard paraffin imbedding and sectioning methods resulted in much trichomonad distortion. Flagella stained poorly or not at all, and structural details could not be recognized in any stain. The general ovoid or pear shape, however, was preserved. The trichomonads could usually be distinguished from tissue or blood cells by their shape, lack of nuclear detail, vacuolated cytoplasm, and occasional, somewhat distorted, flagella.

Following is a description of both gross and microscopic pathology as observed at the different stages of lesion development following infection.

SECOND DAY

Gross: One of the birds showed fine, white, punctate spots on the mucosal surface of the pharynx around the pharyngeal papillae. Another presented an accentuation of the subcapsular vessels in the liver. No other changes were noted.

Microscopic: No actual ulcers were found in the oropharynx, but organisms were recognizable on the mucosal surface, especially in the pharyngeal papilla region. The trichomonads were arranged side by side, perpendicular to the surface, where they formed a layer on the squamous epithelial surface

resembling columnar epithelium (Figs. 1, 2). No inflammatory reaction was seen in the subjacent epithelium or in the underlying connective tissue, except near the gland openings. There the submucosa showed a mild mononuclear reaction. The organisms were further identified in the gland lumina and adjacent to small capillaries near the glands. Their pear shape was apparent, but flagella, axostyles, and other structures were rarely identifiable. Vacuoles and PAS-positive granules were noted in the cytoplasm.

In the liver, recognition of the earliest lesions was made difficult by the presence of lymphoid patches about the sublobular portal veins. These patches were also noted in the control birds, and the absence of necrotic or other changes in the surrounding hepatic cells made us consider them normal. (We can find no detailed description in the American literature of the normal histological structure of the pigeon liver.)

Fig. 1. Pharynx, with intact squamous epithelium showing the individual trichomonads arranged perpendicularly to the surface. Second day. Hematoxylin and eosin. ×200.

No definite abscesses or other evidence of trichomonad dissemination were seen in the liver or in other organs at this stage of infection. Trichomonads were not identifiable in the liver sinusoids.

THIRD DAY

Gross: The oropharynx was partially covered by a thin mucous film. The liver revealed multiple tiny white or whitish-yellow, well demarcated, solid nodules that measured up to 0.2 cm. These were distributed throughout all lobes. One bird showed several small, well circumscribed abscesses, measuring up to 0.4 cm, near the hilar region of both lungs. They were whitish-yellow and were situated around the secondary and tertiary bronchi. The abscesses were solid, with soft, but not liquid, centers.

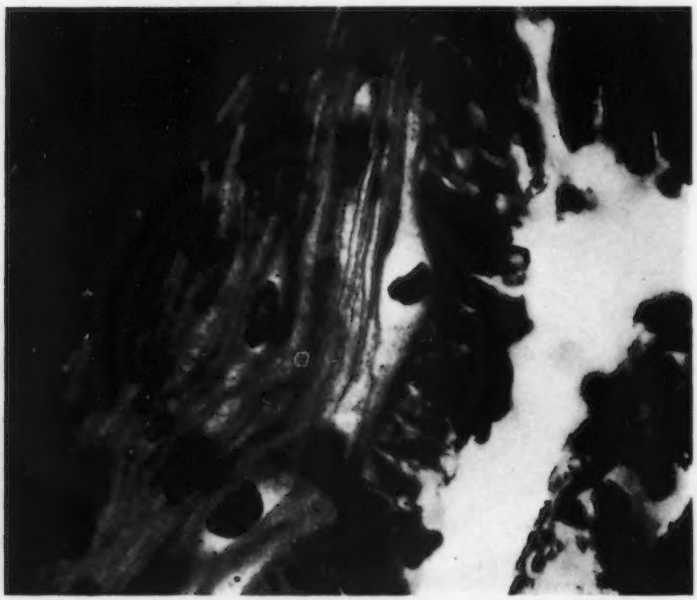

Fig. 2. Area at left of Fig. 1, magnified further. Parasites are seen as "attached" to epithelial surface. Note poor nuclear detail and vacuolization of the trichomonads. Hematoxylin and eosin. ×400.

Microscopic: The pharynx at this stage revealed occassional shallow ulcers. At the ulcer edges the epithelial surfaces were disrupted by intra-epithelial parasites that seemed to be causing separation of the squamous epithelial cells. A heavy infiltration of leucocytes was noted in the submucosa around the glands, especially in the pharyngeal and laryngeal regions. Many organisms were seen around the dilated vessels, but no actual penetration of the vessels was noted. One bird showed the palisading of trichomonads on the surface of the tracheal epithelium, as well as numerous shallow tracheal ulcerations. This same pigeon had the lung abscesses noted above. These lung lesions showed necrotic centers circumscribed by lymphocytes, palisading mononuclear cells, and rare giant cells. The trichomonads lay between the necrotic centers and the peripheral normal lung tissues. Aspiration of parasites from the mouth or tracheal lesions could have been the origin of the lung lesions.

The earliest liver lesions were recognized at this stage as focal necroses with inflammatory reaction. The latter was characterized by mononuclear cells and heterophiles. The abscesses were situated in all zones of the lobules. The liver reticulum within the abscesses was not totally destroyed. It was, indeed, better preserved than the hematoxylin and eosin sections would have suggested. Trichomonads were difficult to identify.

FOURTH DAY

Gross: At this stage the mucosal surface of the pharynx was covered by an uneven, yellow pseudomembrane intermingled with food particles. No gross ulceration was evident. The liver showed an increase in size of the abscesses, with lesion coalescence, some lesions measuring up to 0.4 cm. They possessed a homogeneous, firm structure, with a slightly yellowish tinge.

Microscopic: The pharynx showed mucosal ulcerations. The glands had disappeared in the massive inflammatory reaction, and the degenerated glandular cells, plus necrotic material, were found in the deep portions of the pharyngeal wall. This inflammatory process extended throughout the sub-

mucosa and was covered, in the margins of the ulcer, by an overhanging hyperplastic epithelium. The trichomonads were recognized only near the surface, intermingled with the exudate. The peculiar parasite palisading was present throughout the pharynx wherever the squamous epithelium was intact (Figs. 1, 2). Other small ulcerations were noted near the gland openings. In the nonulcerated area the epithelium was slightly hyperplastic.

The liver now revealed large, more advanced lesions (Fig. 3). The necrotic areas showed dead liver cells surrounded by heterophiles and mononuclear cells. Here the trichomonads were best identified following staining in Heidenhain's hematoxylin. The reticulum was still present in these lesions, though seriously disrupted.

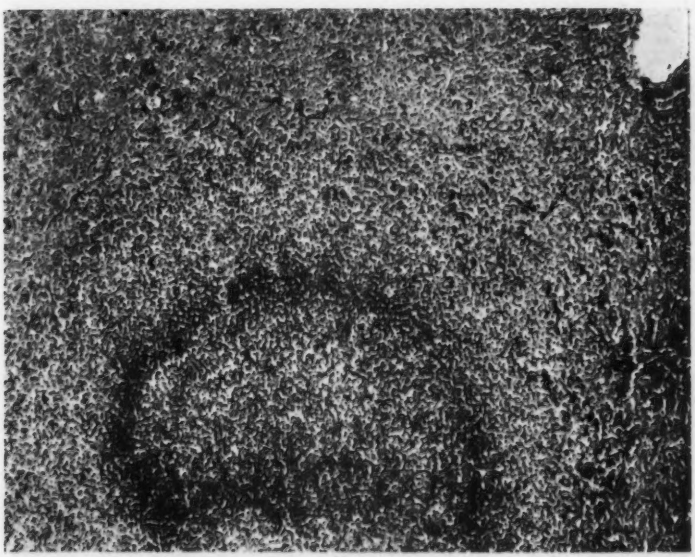

Fig. 3. Liver abscess. Organisms were most numerous at the junction between liver cells and leucocytic infiltration, but cannot be distinguished at this magnification. Fourth day. Hematoxylin and eosin. ×80.

FIFTH DAY

Gross: Other than an increase of congestion of the mucosal surface, the mouth showed no change from the 4th day. The liver lesions were larger, a deeper yellow, and measured up to 1.0 cm.

Microscopic: The pharyngeal ulcers (Figs. 4, 5) were deeper than those of the 4th day. The parasites were close to the dilated and congested vessels, but no intravascular trichomonads were seen.

The liver lesions were essentially similar to, but larger than, those of the 4th day.

SEVENTH DAY

Gross: No deep ulcers were noted in the oropharynx, but heavily congested submucosal vessels were seen below the

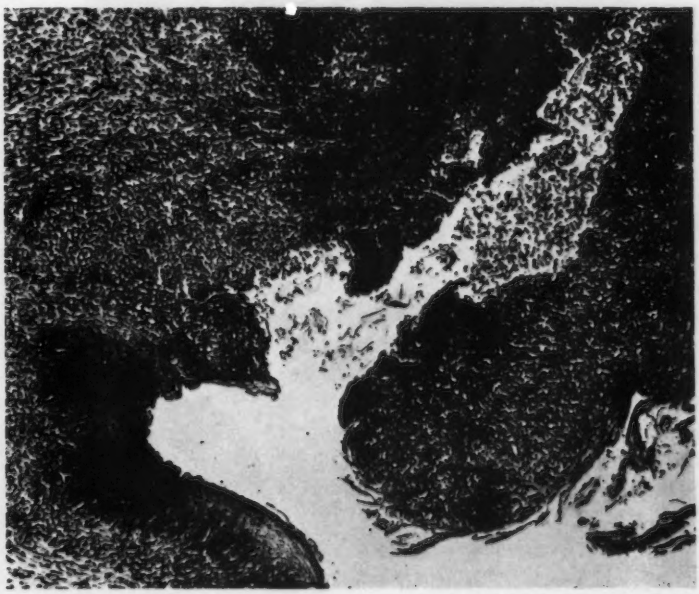

Fig. 4. Ulceration in pharynx. The center of the ulcer is formed by necrotic purulent exudate (at upper left) surrounded by a mononuclear inflammatory response. Fifth day. Hematoxylin and eosin. ×80.

pesudomembrane. The liver abscesses were fewer, but confluent and larger. They showed irregular and well delineated borders, measuring up to 2.0 cm. No rupture of the abscesses onto the peritoneum was noted, even in this advanced stage. The lesions were still solid, not liquid, with a pronounced yellow color.

Microscopic: The ulcerations in the mucosa were larger, and in certain areas the organisms were very close to the vessels. The areas of intact epithelium still showed the mucosal palisading of the trichomonads.

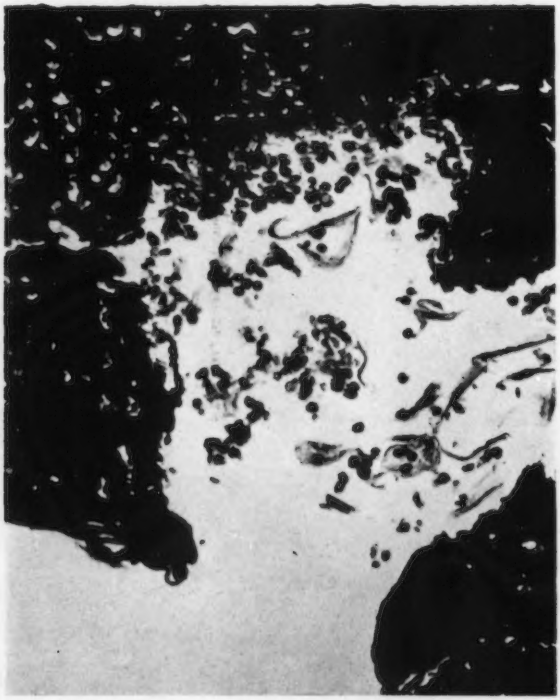

Fig. 5. Area near center of Fig. 4, magnified further. The trichomonads may be recognized in the center and upper left of the ulceration. Many more were found in the dense exudate. Hematoxylin and eosin. ×200.

The liver reticulum showed fragmentation, with some complete reticulum destruction. The centers of the abscesses contained necrotic cellular debris, with no intact hepatic cells. Near the abscess periphery the tissue swarmed with trichomonads. The liver capsule was thickened over the abscesses. Some of the larger lesions seemed to be partially made up of infarct-like areas of necrosis, where ghosts of hepatic cells were plainly seen. In such areas the organisms were particularly numerous and, in the absence of leucocytes, easily recognized in all stains used.

At death of pigeon (5th to 10th days). Birds autopsied after death during the natural course of the infection consistently showed caseous-like hepatic lesions similar to those described from the birds sacrificed on the 5th and 7th days. Though no significant visceral lesions were found other than those mentioned above, one of us (RMS) had noted, during many years of passage of this organism through healthy nonimmune pigeons, the occasional occurrence of lesions similar to the above hepatic ones in the lungs, pancreas, myocardium, brain, kidney, and the various air sacs.

DISCUSSION

As mentioned, the pathological findings in birds infected with the protozoan flagellate, *Trichomonas gallinae*, have not been studied extensively. Yet the fulminating and fatal disease that is produced by some strains in domestic pigeons, doves, and occasionally in other species of birds, in contrast with the almost commensal relationship produced by other strains in the same type of bird, makes the pathological aspect of considerable interest. The present studies indicate that, following infection of the oropharynx, the organisms multiply locally on the mucosal surface and in the secretions. The peculiar mucosal palisading of the trichomonads is of special interest, and in some way not yet understood may result in eventual surface ulceration. This packing of the cell surface was noted by Stabler[6] on isolated epithelial cells found in the saliva of infected pigeons.

The trichomonads also appear able to migrate to the depths of the pharyngeal mucous glands, and by this route

penetrate the underlying tissues. Presumably, having once invaded the tissue beneath the pharyngeal mucosa and the surrounding mucosa glands, the organisms rapidly reach the liver. We would surmise that the route of spread is via the blood.

The pigeon liver is peculiarly susceptible to the Jones' Barn strain of *T. gallinae*. The rarity of other visceral lesions bears this out. The one case in the present study that spread to the lungs seems clearly to have been the consequence of tracheal extension and ulceration, followed by aspiration into the lungs.

The basic pathological response is of purulent inflammation. Why no liquefaction had occurred in the centers of the necrotic areas is interesting. Grossly, the lesions resemble caseation necrosis, but microscopically the inflammatory cells are at least half heterophiles. In monocytic and lymphocytic cell exudates, as in so-called granulomatous inflammatory diseases in man, liquefaction is absent and caseation typical. This is because of the low concentrations of proteolytic enzymes in these mononuclear inflammatory cells, in contrast with that in neutrophilic polymorphonuclear leucocytes of man. Pigeons have a circulating white-cell count of about 54% lymphocytes, 35% eosinophils or pseudoeosinophils, 4% basophils, and only some 4% heterophiles. Perhaps an exudate of such proportions cannot result in liquefaction. It should be noted that, in spite of the gross appearance, a tuberculoid picture was never seen microscopically. The mononuclear reactions at the periphery of the lesions were never epithelioid, and the rare giant cell formation did not alter the characteristic abscess-like picture.

The trichomonads were not visible in the centers of the necrotic areas, but were consistently recognizable at the periphery of the lesions in the liver and pharynx. Occasionally they were seen in enormous numbers in the older lesions, being especially abundant in the infarct-like areas. In the paraffin sections cut at 6 μ, organisms were difficult to find early in the development of the hepatic abscesses. Thinner sections made recognition easier, but the flagellates still appeared

blurred, fragmented, and indistinct as compared to tissue cells. Apparently the trichomonads are far more fragile than host cells and are distorted by standard paraffin histological techniques.

The extraordinary rapidity of the development of the abscesses and tremendous multiplication of the organisms within 5–7 days is noteworthy. The promptness of the necrosis suggests that hypersensitivity plays no role. It does suggest that the Jones' Barn strain of *T. gallinae* possesses a hepatic toxicity in the nonimmune pigeon comparable with that of *Staphylococcus aureus* in man. Studies on the strain differences in this trichomonad are indicated, as well as are experiments on immunologic responses. The pigeons appear to die as a result of massive hepatic destruction.

SUMMARY

Nonimmune domestic pigeons were infected with the highly virulent Jones' Barn strain of *Trichomonas gallinae*, and sacrificed at intervals for histologic study. Others were autopsied after death during the natural course of the disease. Lesions were confined almost entirely to the oropharynx and liver. Ulcers occurred in the pharynx following peculiar palisading of the parasites on the surface. Progressively enlarging, non-liquefying abscesses in the liver appeared to be the cause of death. Failure of trichomonad-induced abscesses to liquefy, therefore appearing as caseating lesions, may be the consequence of the low neutrophilic polymorphonuclear leucocyte count in pigeons (about 4%).

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CASE REPORT—*DISPHARYNX NASUTA* CAUSING SEVERE PROVENTRICULITIS IN PIGEONS

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CRAM[5] reported an outbreak of *Dispharynx spiralis* (Syn. *D. nasuta*) infestation in a flock of Racing Homer pigeons of the U. S. Army Signal Corps, Fort Sam Houston, Texas. The report did not describe the microscopic changes in the infected proventriculus. Raggi and Baker[13] described a natural outbreak, in domestic pigeons in California, of *D. nasuta* and another proventricular worm, *Tetrameres americana*. These authors believed that *T. americana* was primarily responsible for the moribund condition of the birds, and the other parasite was not significant.

D. nasuta was originally described by Rudolphi,[14] in 1819, as a parasite of the sparrow, under the name of *Spiroptera nasuta*. This nematode has since been reported from the domestic fowl (Diesing,[7] Casali,[2] Colucci,[3] Piana,[11] Johnston,[9] Purvis,[12] and others) and from several wild game birds (Cram,[4] Madsen,[10] Boyd,[1] Goble and Kutz,[8] and others). In

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the United States, *D. nasuta* has been reported as a pigeon parasite in some Eastern seaboard, Midwestern, and Southern States.

Fig. 1. Proventriculus opened to show adult *Dispharynx nasuta in situ.* ×6.

Recently, one of us (DSJ) encountered a natural infection of *D. nasuta* in domestic pigeons near Dade City, Florida. This paper reports the finding, the histopathology, and facts on the life history and control of the parasite. The authors believe this report is the first natural *D. nasuta* infection in pigeons from Florida, and one of the few in which the infestation was associated with pathological changes and death.

The infected flock was 50 White King and Blue King pigeons. The birds were not confined but free to wander and eat at will. Within a few weeks of diagnosis, 15 birds, from a few months to 4 years old, had died, presumably from *D. nasuta* infection. Hawks were reported to have killed 20 additional birds, which may have been easy prey because of weakness.

Fig. 1 shows the proventriculus of one of the pigeons examined. A large number of worms were embedded in the greatly thickened mucosa. These gross observations agree with those of Cram,[5] who observed that the proventriculus was greatly enlarged and the tremendously thickened mucosa

Fig. 2. Section of proventriculus showing: (a) portions of worms in desquamated area, (b) papillary proliferation, and (c) marked vascular congestion of mucosa. ×40.

was covered with a necrotic mass containing many nematodes. She also reported that infection signs were droopiness, loss of weight, a ravenous appetite even until death, and anemia.

Histopathological findings. One of us (WTS) noted that many adult worms were embedded in the mucosa of the diseased proventriculus, but none had penetrated as far as the muscular tunic. In most instances, the sections of worms were surrounded by a myxomatous-like exudate that was essentially acellular. The exudate was probably of mucous origin intermixed with strands of fibrous tissue.

Marked vascular congestion was associated with a few infection sites (Fig. 2c, 3a). The reaction was essentially proliferative in type, with a minimal inflammatory cell reaction (Fig. 3). A large part of the superficial mucosa had been desquamated (Fig. 2-a); in some areas that still had intact mucosa, there was papillary proliferation (Fig. 2-b) and edema of the tunica propria. Several pink-staining masses,

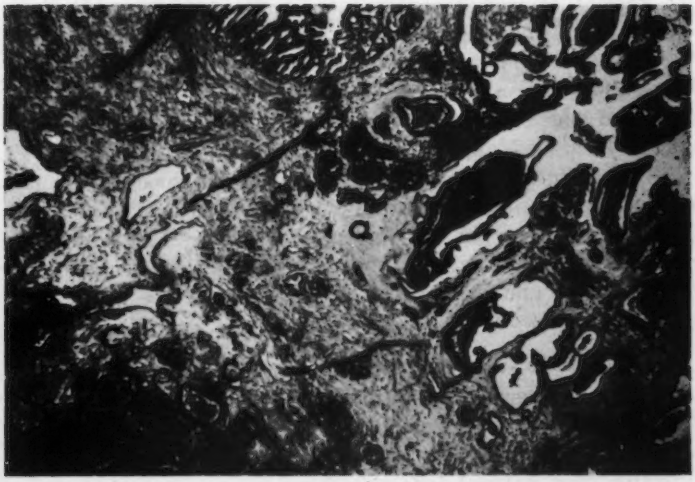

Fig. 3. Section of mucosa illustrating general proliferative type of reaction with a minimal inflammatory cell infiltration: (a) area of marked vascular congestion, (b) group of endothelial-lined vessels containing erythrocytes, and (c) glandular lumena filled with pink exudate. ×40.

superficial and as large as 3 mm in diameter, contained foci of rod-shaped bacteria. These cellular masses were discrete but not encapsulated; although granules were not distinct, it was believed many cells were granular leukocytes, along with epithelial cells undergoing early necrotic changes. There was an outer zone of readily identifiable heterophiles surrounding eosinophilic cellular masses. These masses probably represented a tissue reaction to secondary bacterial invaders established after the parasites had caused a primary traumatic lesion (Fig. 2, 3).

Briefly, the infected proventriculi showed primarily a catarrhal type of inflammation characterized by epithelial desquamation, papillary proliferation, hypersecretion of mucus, congestion, and secondary bacterial invasion of the superficial mucosa.

Life cycle. *D. nasuta* requires an intermediate host for its complete development. Cram[6] fed infective eggs of this nematode to various invertebrate hosts and was able to infect two species of isopod crustaceans—pillbugs (*Armadillidium vulgare*) and sowbugs (*Porcellio scaber*). The development of the larvae to the infective stage was completed within 26 days. When the intermediate host was ingested by bobwhite quail and pigeons, the infective larvae developed into adult worms within 27 days.

Treatment. No drug is known that will successfully remove this proventricular worm from its avian host. Since the eggs pass out in the droppings of infected birds, control measures must be directed toward disposal of the excreta of such birds, and elimination of the intermediate hosts with appropriate insecticides.

SUMMARY

Severe proventriculitis due to *Dispharynx nasuta* infection was described in pigeons. Reports of this nematode in domestic and wild game birds have been increasing, which indicates that natural infection is more common than generally believed. In determining the causes of clinical para-

sitism in domestic or wild game birds, the importance of this proventricular worm should not be neglected.

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SOME FACTORS AFFECTING ROUS SARCOMA VIRUS ACTIVITY IN THE EMBRYONATED EGG INOCULATED BY THE YOLK-SAC ROUTE*

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VIRUSES were first suggested as the cause of tumors by Borrel[8] and Boss.[11] The first definitive evidence, however, that a filterable agent was involved in the neoplastic process was reported by Ellerman and Bang.[28] In 1910, Rous[49] described a spindle-celled sarcoma of a Barred Plymouth Rock hen. He later reported[50,51] that this disease could be transmitted with a cell-free filtrate. Since then the characteristics and properties of Rous sarcoma and its etiological agent, the Rous sarcoma virus (RSV), have been studied extensively, and their value as tools in tumor research has been recognized.[5,21,32,41]

Several methods have been reported for the bioassay of RSV, and many of the earlier methods, or modifications thereof, are still in use. Many researchers[1,12,13,22,31,51,52,53] used as evidence of virus activity the presence or absence and/or relative size of tumors produced on the chicken following inoculation of RSV at multiple sites intramuscularly, subcutaneously, or intracutaneously. Since metastases to the site of injury produced by a noninfective inoculation were known to

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have occurred, the multiple-site technique for virus bioassay was abandoned by Carr and Harris[19] in favor of one inoculation site per chick. Kenzy[34] described a method of bioassay for RSV using one inoculation site per chicken, and employed the method of Reed and Muench[48] for interpolating the greatest dilution capable of producing a tumor in 50% of the inoculated chicks (ID_{50}). Groupe *et al.*[29] observed a characteristic hemorrhagic lesion in the meninges and subsequent death, found to be directly correlated with the dose of RSV inoculated. Since the early report of Keogh[37] that the chorioallantoic membrane (CAM) can be infected by RSV with subsequent ectodermal proliferation, no mention was made of using this method for bioassay of RSV until Rubin[54,55,56] reported a linear correlation between virus concentration and the number of lesions produced. A number of improvements on the CAM method have been reported recently.[30,46,47]

Excellent results with RSV bioassays that use the hemorrhagic lesion[24,42] as evidence of infection have been reported by a number of investigators.[4,9,10,37] Within the past decade, scientists have reported on the growth of RSV on adult chicken fibroblasts[39,43,58,65] or chicken embryo cells *in vitro*, with the production of characteristic cytopathic effects. More recently, bioassays of RSV using tissue culture methods have been described.[40,57,62]

Little is known concerning the use of the embryonated egg yolk-sac method for inoculation of RSV. Inoculation of the yolk sac is very simple, rapid, and relatively safe. Various workers[7,33,61] reported successful transplants of mouse and rat tumors by this method, but others,[16,63] working with various mouse, rat, and avian tumors, reported a very small percentage of successful transplants.

Andrewes[2] reported that birds carrying the Rous sarcoma may develop neutralizing antibodies in their sera against RSV, but found that the response was very inconsistent. He also noted that Rous virus neutralizing antibodies (RVNA) were detected in apparently "normal" fowl. Several years later Andrewes[3] reported the presence of RVNA for a maxi-

mum period of 2 weeks in the sera of chicks from hens whose sera also showed neutralizing activity.

The report of Duran-Reynals[24] that the vast majority of adult chickens living under ordinary conditions possessed specific RVNA, instigated a series of investigations on the relationship of age of the fowl to the presence or absence of RVNA. Duran-Reynals and King[26] found that immune bodies in the blood of aging chickens neutralized the viruses of both Rous sarcoma in the chicken and its duck variant. They hypothesized that resistance of chickens against these viruses was linked with the presence of humoral factors. On the other hand, comparable immune bodies from aging ducks neutralized only duck tumor viruses and not the chicken tumor viruses. In this instance, it was theorized that the refractory state of ducks against heterologous chicken viruses could be linked not to humoral factors but rather to species resistance.

A possible antigenic relationship between lymphomatosis and RSV was suggested by Duran-Reynals *et al.*[27] When it was observed that sera from chickens hatched and reared in isolation, but showing an absence or low incidence of lymphomatosis, were devoid of RVNA. Sera from genetically related chickens reared in contaminated surroundings, in contrast, showed a high incidence of lymphomatosis and RVNA.

Consistent with this, Duran-Reynals and Freire[23,25] found that in chickens and ducks of widely varying ages bearing sarcomas, lymphocytomas, and other tumors, the RVNA titers increased substantially during a period of 5 months. In a survey of field flocks, Kenzy and Neuzil[36] found RVNA in over 60% of the sera from flocks with 30–50% losses from fowl lymphomatosis. In flocks where losses from this disease were less than 5% the incidence of RVNA was less than 10%. Kenzy[35] further emphasized the complexity of the problem when he demonstrated the presence of RVNA in chickens inoculated with a myxosarcoma, and the subsequent deaths of many of these with visceral lymphomatosis.

The following experiments were carried out 1) to investigate the yolk-sac method and evaluate it as a tool for bioassay of RSV and detection of RVNA; 2) to determine

the neutralizing effects of various concentrations of serum containing RVNA on the activity of RSV when inoculated into the yolk sac of embryonated eggs; and 3) to compare the activity of RSV inoculated into the yolk sac of embryonated eggs from hens having RVNA in their sera, and from isolated control hens without demonstrable RVNA.

MATERIALS AND METHODS

Stock RSV. Tumor desiccate was originally obtained from Dr. F. Duran-Reynals, Yale School of Medicine, New Haven, Connecticut, through Dr. G. E. Cottral, Regional Poultry Research Laboratory, East Lansing, Michigan. Stock cell-free RSV was prepared as follows: RSV was serially passed 3 times by inoculation into the left pectoral muscle of 2-week-old chicks. The final passage was harvested, comminuted, partially purified by differential centrifugation,[14] and sealed under partial vacuum (22 cm Hg) in 2.0-ml quantities.[15] It was then quick-frozen in an alcohol-dry ice mixture and stored in a carbon-dioxide-ice chest for future use. The activity of the material was assayed in 2-week-old chicks by subcutaneous inoculation, one site per chick.[34] The reciprocal of the logarithmic dilution infecting 50% of the test chicks (ID_{50}) was calculated and found to be approximately 5.40.

Fertile hen eggs. Three flocks (Nos. 1, 2, and 3), Regional Poultry Research Laboratory (RPRL) Line 15, of White Leghorn hens were maintained as sources of fertile eggs for these experiments. This particular strain has been inbred and selected toward susceptibility to avian lymphomatosis at the RPRL, East Lansing, Michigan, for the past 21 years without the introduction of any outside stocks of chickens.[66,67] Flock No. 1 consisted of chickens hatched from fertile eggs obtained from the RPRL in 1957. Flock No. 2 consisted of progeny from flock No. 1, being about 11 months younger. These flocks were hatched, reared, and maintained under isolated conditions, with precautions to prevent introduction of contaminated materials. Except for special circumstances (bleeding, cleaning, etc.), the authors were the only people who entered the laying pens. Flock No. 3, also progeny of

Flock No. 1, consisted of chickens hatched from embryos surviving preliminary experimental yolk-sac inoculations of RSV, and RSV-aerosol transmission studies. About 75% of the hens of this group exhibited RVN in their sera when tested at various intervals during lay.

Eggs were collected daily from the laying flocks and held in storage at 7–10°C for 10–12 days. In a given experiment if enough eggs were obtained in a 10-day collection period, these were set. In most instances, however, a 12-day collection period was necessary.

Sera containing RVNA. Sera containing RVNA were produced in the following manner: Two-week-old chicks were experimentally infected with RSV by wing-web inoculation, and examined weekly for tumor development and subsequent tumor regression. Only chickens exhibiting regressing tumors were bled (weekly intervals) for serum collection. The sera were pooled, tested for anti-Rous virus activity, and frozen (–20°C) for future use.

Bioassay of viral activity. Fertile eggs were incubated in a commercial egg incubator (Jamesway, Model 252B, James Manufacturing Co., Fort Atkinson, Wisconsin) set at 37.5–38.0°C and about 62% relative humidity. After the desired time of incubation, depending on the experiment, the embryonated eggs were inoculated via the yolk sac, using the method of Beveridge and Burnet[6] as follows: 1) the position of the embryo was located by candling and a hole was drilled or punched through the shell at the air-cell end of the egg; 2) a 20-gauge 1¼-inch hypodermic needle was inserted to its maximum depth adjacent to the embryo, and the inoculum (0.1 ml) injected into the yolk sac; 3) the hole in the shell was sealed with collodion, and the embryonated egg was re-incubated. It has been reported that as much as 1.0 ml can be inoculated into the yolk sac without injuring the embryo.[6]

The inoculated eggs were candled daily and examined if deaths were observed. On the 19th day of incubation, all surviving embryos were transferred to a hatcher. All hatched chicks were sacrificed and examined when 3 days old.

Infection with RSV was judged by macroscopic evidence

of neoplastic growth on the amnioallantois or on the periphery of the umbilical opening and/or hemorrhagic lesions. Embryonated eggs or chicks having either or both types of lesions were considered infected with RSV. Deaths before the 8th day postinoculation were disregarded, because: 1) they could have been due to factors other than RSV; 2) lesions were not observed consistently within this time period, even with high concentrations of RSV.

Influence of embryo age on nonspecific deaths. To compare deaths from inoculation injury or other factors not RSV action in embryos of various ages, 0.1 ml of Simms' solution[59] was inoculated into the yolk sacs of embryonated eggs 4, 6, 8, 10, 12, and 14 days old. The embryonated eggs were candled daily and the number of deaths recorded.

Influence of incubation period on viral activity. Eight- and 10-day-old embryonated eggs were inoculated with a 1:100 dilution of RSV suspension (about 300 yolk sac ID_{50}) to determine the optimum time for development of lesions. Appropriate numbers of such embryos or resulting hatched chicks were sacrificed and examined on the following postinoculation days: 4, 6, 8, 10, 12, 14, and 16.

Effect of RSV concentration. The effect of RSV concentration on its activity in embryonated eggs of various ages was determined as follows: groups of embryonated eggs 4, 6, 8, 10, 12, 14, and 16 days old were inoculated with 0.1-ml of 10-fold dilutions of RSV. The ID_{50} of each group, calculated by the method of Reed and Muench,[48] was compared.

Influence of flock age on embryonated egg response to RSV. To compare the activity of RSV in embryonated eggs laid by hens of 2 different age groups (about 11 months difference in age: flocks 1 and 2), fertile eggs were collected from these flocks, incubated 10 days, inoculated with 10-fold dilutions of RSV in parallel series, and followed as described. The reciprocals of the logarithmic dilutions of the ID_{50} of virus were calculated and compared.

The activity of RSV in the embryonated eggs from 3 different flocks. The activity of RSV was compared in embryonated eggs from the following 3 strains of White Leghorn

hens: 1) RPRL Line 15; 2) commercial breeder; and 3) Department of Poultry Science, Washington State University (WSU). Ten-day-old embryonated eggs were inoculated with 10-fold dilutions of RSV, and the reciprocals of the ID_{50} of virus were calculated.

Effect of RVNA on RSV activity in the embryonated egg. A study of the potency of serum containing RVNA was made by the block titration technique. Mixtures of equal quantities of 10-fold dilutions of serum containing RVNA, and 10-fold dilutions of RSV, were allowed to incubate overnight (12–15 hours) at refrigerator temperatures (4–7°C), and inoculated the following morning into the yolk sacs of 10-day-old embryonated eggs. The logarithmic dilution of the ID_{50} of virus was calculated and compared for each dilution of RVNA.

The feasibility of detecting neutralizing antibodies in chicken serum by the yolk-sac inoculation of embryonated eggs with mixtures of serum and RSV was determined as follows: A 1:2 dilution of serum containing RVNA was mixed with equal quantities of relatively concentrated suspensions of RSV (1:100 in trial No. 1, and 1:200 in trial No. 2). The mixtures were held overnight at refrigerator temperatures, and inoculated into the yolk sac of 10-day-old embryonated eggs the following morning.

Influence of RVNA in hen sera on susceptibility of their eggs to Rous sarcoma virus. The activity of RSV in embryonated eggs from hens exhibiting RVNA (flock No. 3), as compared to the activity in embryonated eggs from isolated

Table 1. Deaths from injury or non-specific causes occurring within the first 7 days of inoculation into various-aged embryos.[1]

Age of embryo (days)	No. deaths at days postinoculation:						Total deaths and %
	2	3	4	5	6	7	
4	3	3	1	1	1	1	10/32(31)
6	5	3	1	0	1	1	11/24(44)
8	4	1	1	0	0	1	7/29(24)
10	2	0	0	0	0	0	2/26(8)
12	1	0	0	0	0	0	1/30(3)
14	1	0	0	0	0	0	1/26(4)

[1] The yolk sacs of embryonated eggs were inoculated with 0.1 ml of Simms' physiologic solution.[54]

control flocks (flock Nos. 1 and 2), was determined as follows: Series of parallel titrations were run in 10-day-old embryonated eggs from these 3 flocks, and the reciprocal of the logarithmic dilution of the ID_{50} of virus was calculated.

RESULTS

Effect of embryo age on nonspecific deaths. Deaths from inoculation injury or other factors not RSV action in embryos of various ages were compared. As the embryo age increased to 10 days the number of nonspecific deaths was significantly decreased (Table 1). Chi-square analyses ($\chi^2_{.10(1)} = 2.7$) showed significant differences between embryos of the following ages (calculated chi-square values were adjusted according to Snedecor[60]) : 4- and 10-day-old ($\chi^2 = 3.51$), 6- and 10- day-old ($\chi^2 = 7.03$), and 6- and 8-day-old ($\chi^2 = 3.81$). No

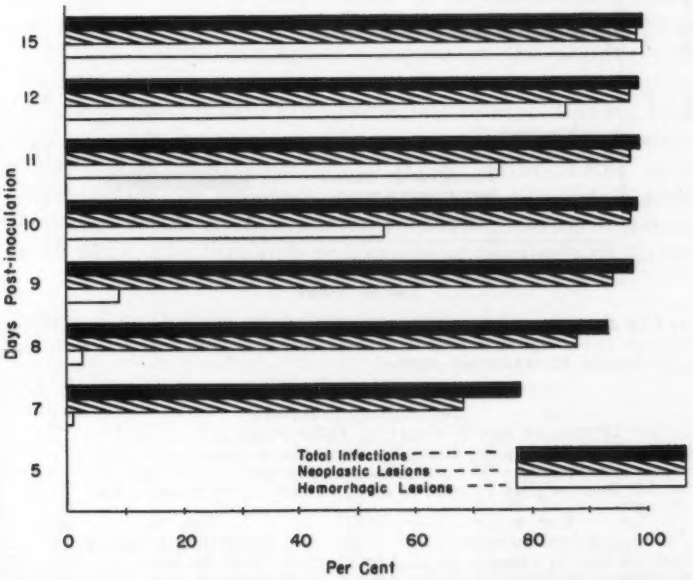

Fig. 1. Per cent of lesion types (calculated from accumulated totals, Reed and Muench[48]), observed at various postinoculation intervals in embryonated eggs inoculated into the yolk sac with Rous sarcoma virus when 10 days old.

significant differences were noted between 4- and 8-day-old and 8- and 10-day-old embryonated eggs. It was also noted that 23 of 32 (about 72%) of the deaths occurred during the first 72 hours after inoculation, and 28 of 32 (about 87%) were recorded in the 4- to 8-day-old age range.

Influence of incubation period on virus activity. The earliest manifestation of activity of RSV injected into the

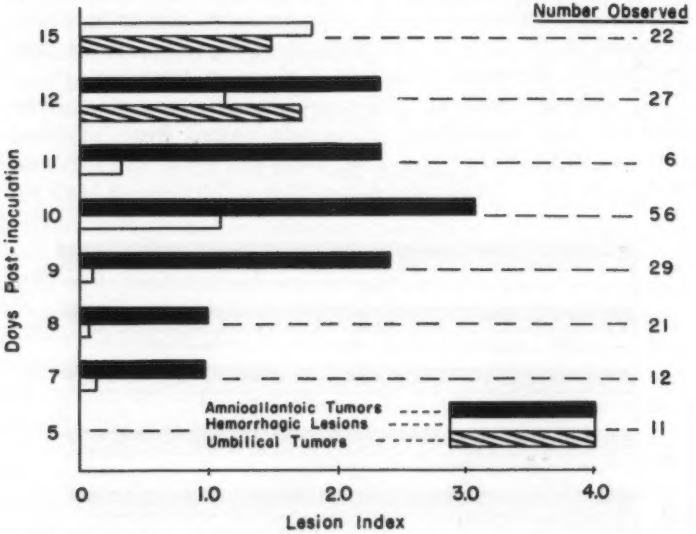

Fig. 2. Average severity index of lesion types observed at various intervals following inoculation of Rous sarcoma virus into the yolk-sac of 10-day-old embryonated eggs.

Key to amnioallantoic tumors

10 mm. or less in diam; no hemorrhage	1.0
10 to 20 mm. in diam; no hemorrhage	2.0
Greater than 20 mm. in diam; moderate hemorrhage	3.0
Greater than 20 mm. in diam; severe hemorrhage	4.0

Key to umbilical tumors[1]

5 mm. or less in diam	1.0
5 to 10 mm. in diam	2.0
10 to 15 mm. in diam	3.0
Greater than 15 mm. in diam	4.0

Key to hemorrhagic lesions

1 to 5 focal lesions	1.0
5 to 10 focal lesions	2.0
10 to 20 focal lesions	3.0
More than 20 focal lesions	4.0

[1] In hatched chicks only.

yolk sac appeared to be a proliferation of neoplastic cells in discrete focal areas on the amnioallantois. Cell proliferation on the amnioallantois at this site was probably due to deposition of virus in this membrane during passage of the needle to the yolk sac. Since a separate needle was not used for each embryonated egg, it is anticipated that virus would be deposited in or on membranes and into cavities along the path of inoculation. However, it was interesting to find that the focal area of neoplastic tissue on the amnioallantois was invariably observed in the same area in relation to the position of the developing embryo.

Macroscopic lesions first appeared about 7 days after inoculation. Embryos sacrificed on subsequent days exhibited more severe lesions on the amnioallantois. During the later phases of embryonic development, hemorrhagic lesions usually appeared in the liver, occasionally in the heart and gizzard, and rarely in the subcutaneous tissues. Infected hatched chicks invariably exhibited neoplastic lesions at the periphery of the umbilical opening.

These observations are summarized in Figs. 1 and 2. Both neoplastic and hemorrhagic lesions were first observed about 7 days after inoculation of 10-day-old embryonated eggs. Subsequently, the incidence and severity of neoplastic lesions increased as the postinoculation incubation period increased to 10 days. The incidence and extent of hemorrhagic lesions

Table 2. Rous sarcoma virus infections observed in embryonated eggs sacrificed at various postinoculation intervals.[1]

Postinocu. time (days)	Age when embryonated eggs inoculated			
	8-day-old		10-day-old	
	No. positive	Per cent infection	No. positive	Per cent infection
4	0/17	0.0	0/16	0.0
6	2/27	12	12/19	63
8	10/17	59	34/37	92
10	21/23	91	34/35	97
12	21/29	72	24/26	92
14	18/19	95	35/36	97
16	21/22	96		

[1] Infections based on tumorous growth on the amnioallantois or umbilicus, and/or hemorrhagic lesions.

Table 3. Effect of Rous virus concentration on response of the embryonated 10-day-old egg inoculated via the yolk sac.

Reciprocal of log. dilution	No. Inoc.	Reactions of embryo deaths[1]		Reactions of hatched chicks[2]		Total no. positive[3]	Accumulative infections[4]	Per cent infections
		+	—	+	—			
2	15	7	0	3	2	10/12	43/45	96
3	15	7	0	8	0	15/15	33/35	94
4	15	1	0	10	2	11/13	18/22	82
5	15	0	3	7	3	7/13	7/17	41
6	16	0	1	0	15	0/16	0/26	0
Controls[5]	14	0	2	0	10	0/12		

Reciprocal log. dilution ID_{50}[4] $= 4.0 + \frac{81.8 - 50.0}{81.8 - 41.2} = 4.0 + \frac{31.8}{40.6} = 4.0 + 0.783 = 4.783$

[1] Necropsied embryos showing amnioallantoic tumors and/or hemorrhagic lesions were considered positive.

[2] Hatched chicks were sacrificed on the 3rd day post-hatching. Those showing umbilical tumors and/or hemorrhagic lesions were considered positive.

[3] Numerator = total number positive; denominator = number observed; deaths before the 8th day postinoculation were disregarded in the final analysis; thus, the apparent discrepancy with numbers inoculated.

[4] Accumulated totals and ID_{50} calculated by the method of Reed and Muench.[43]

[5] Controls were inoculated with 0.1 ml of Simms' physiologic solution.[54]

increased more slowly, reaching its maximum at 15 days (4-day-old chicks), the longest period of observation.

A highly significant difference was found between 8- and 10-day-old embryos examined on the 6th or 8th day following inoculation (Table 2). Only 12% of embryos inoculated when 8 days old showed lesions on the 6th day following inoculation, whereas 63% of embryos inoculated when 10 days old were infected in the same period ($\chi^2 = 7.9$). On the 8th day following inoculation of RSV, embryos inoculated when 8 days old showed only 59% infection, whereas those inoculated when 10 days old showed 92% infection ($\chi^2 = 6.4$). When, however, the sacrificing time was delayed to 10 days or later, no significant differences were observed. Tissue response appeared to be significantly more rapid and comparatively more severe in 10-day-old embryos than in 8-day-old embryos.

Table 4. Effects of Rous sarcoma virus concentration on its activity in 8- and 10-day-old embryonated eggs.

Age of embryo (days)	Trial no.	Reciprocal of log. dilution of Rous sarcoma virus					Reciprocal of log. dilution ID_{50}
		2	3	4	5	6	
8	1	4/7[1]	6/8	2/7			3.28
	2	7/7	2/6	0/6			2.73
	3	5/7	3/5	3/6			3.33
	4	6/6	4/8	3/5			3.45
	5	4/5	3/5	3/5			3.57
	6	7/7	2/8	0/5			2.67
	7	7/9	2/6	2/9	0/7		2.82
Total		40/48	22/46	13/43	0/7		3.06[2]
Per cent		83	48	30	0		
10	1	7/7	7/8	3/9			3.67
	2	7/7	8/9	3/10			3.65
	3		7/8	6/9	3/9	0/8	4.42
	4		4/6	3/5	1/6	0/6	4.00
	5	6/7	6/7	7/8	2/6		4.47
	6	12/13	10/16	7/14	7/17	1/18	4.06
	7	10/12	15/15	11/13	7/13	0/16	4.78
Total		42/46	55/69	40/68	20/51	1/48	4.20[2]
Per cent		91	80	59	39	2	

[1] Numerator = number of infections; denominator = number observed; infections based on amnioallantoic or umbilical tumors, and/or hemorrhagic lesions.

[2] Reciprocal of the ID_{50} of virus calculated from totals of 7 separate trials.

Table 5. Effect of flock age on Rous sarcoma virus activity in embryonated eggs

Flock no.	Age (mo.)	Log dilution ID_{50} of RSV[1] Trial no. 1	2	3	4	5	6	Composite ID_{50}[2]
1	19-22	3.56	2.75	2.78	3.18	3.16	3.08	2.87
2	8-11	4.00	—	3.67	—	4.30	3.08	3.60

[1] Reciprocal log. dilution ID_{50} calculated by the method of Reed and Muench.[42]
[2] Reciprocal log. dilution of the composite ID_{50} calculated from totals of the separate trials by the method of Reed and Muench.[43]

Influence of RSV concentration. Table 3 shows the results of a representative titration and the respective endpoint calculations. Since previous studies showed that lesions were not observed consistently until 8 days postinoculation (Figs. 1, 2), embryos dying within 7 days were not considered in the final analysis. Since the embryo age-range between 8 and 10 days was found most susceptibile, embryos of these ages were used for further age-susceptibility studies. The results in Table 4 indicate that 10-day-old embryonated eggs were significantly more susceptible to RSV than 8-day-old embryonated eggs ($\chi^2 = 11.3$ at the 10^{-3} RSV dilution level, and $\chi^2 = 7.5$ at the 10^{-4} RSV dilution level). The composite ID_{50} calculated from the totals of the 7 separate trials showed 3.06 and 4.20 for the 8- and 10-day-old embryonated eggs, respectively. A direct relationship was noted between the concentration of RSV inoculated and the total percent infections produced.

Influence of flock age on embryonated egg response to RSV. Table 5 compares the activity of RSV in embryonated eggs laid by hens of 2 different age groups (flocks 1 and 2). Flock age was a definite factor in the susceptibility of embryonated eggs to RSV. Except for the final trial (No. 6, Table 5) of 4 comparative titrations run, a higher concentration of RSV was required to infect 50% of the embryonated eggs from the older flock (flock 1).

No explanation is offered for the results in trial 6. When all trials were subjected to an analysis of varinace, and compared to the F-distribution ($F_{.05(1,4)} = 7.71$; $F_{.05(5,4)} = 6.26$), a significant difference was observed between flocks ($F_{(1,4)} =$

Table 6. Comparison of the activity of Rous virus in embryonated eggs from 3 sources.

Egg Source	Reciprocal of log. dilution of ID_{50} of virus[1] Trial no. 1	2	3
RPRL—15[2]	4.895	3.646	4.357
Commercial breeder	3.936	3.090	
Poultry science (WSU)[3]			2.725

[1] Infections were based on amnioallantoic or umbilicial tumors, and/or hemorrhagic lesions.
[2] Regional Poultry Research Laboratories, East Lansing, Michigan, strain Line 15, White Leghorn.
[3] Poultry Science Department, Washington State University, Pullman, Washington, (strain) White Leghorn.

11.31) and no significant difference was observed between trials from the same flock ($F_{(5,4)} = 2.24$).

The activity of RSV in embryonated eggs from 3 different source flocks. Embryonated eggs from the RPRL Line 15 White Leghorns were significantly more susceptible to RSV infection than embryonated eggs from the commercial breeder and the WSU flocks (combined accumulated totals from trials No. 1 and 2, 10^{-3} RSV dilution level, Table 6, $\chi^2 = 10.86$). Although no direct comparisons were made between embryonated eggs from the commercial breeder and the WSU flocks, indirect comparisons indicated that embryonated eggs from the commercial breeder line were more susceptible.

Effect of RVNA on RSV activity in the embryonated egg. The composite results of 2 block titration experiments on serum containing RVNA showed that relatively high concentrations of serum were required for a complete neutralization of relatively high concentrations of RSV (Table 7). RSV activity was neutralized almost completely by RVNA serum in a 1:10 dilution, reduced significantly by a 1:100 dilution, and essentially unaffected by a 1:1000 dilution. As the concentration of RSV was increased, so was the concentration of RVNA serum required to neutralize the activity of the virus. As the concentration of RVNA was decreased, the reciprocal of the logarithmic dilution of the ID_{50} of RSV was increased.

Table 7. Neutralizing activity of serum containing Rous virus neutralizing antibodies as compared with negative serum controls.

RSV dilution[1]	Serum dilutions								Neg. serum control	
	10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-1}	
	No. infect.	Per cent infect.	No. infect.	Per cent infect.	No. infect.	Per cent infect.	No. infect.	Per cent infect.	No. infect.	Per cent infect.
10^{-2}	1/14[2]	7.1	12/15	80.0	16/17	94.1	14/15	93.3	12/13	92.3
10^{-3}	0/15	0.0	3/13	23.1	12/17	70.6	9/14	64.3	10/15	66.7
10^{-4}	0/16	0.0	2/14	14.3	7/17	41.2	5/16	31.3	6/13	46.2
10^{-5}	0/14	0.0	2/16	12.5	2/16	12.5	2/12	16.7	3/14	21.4
Log dilution ID_{50} of RSV[3]	<2.00		2.71		3.67		3.52		3.74	

[1] Dilution of Rous sarcoma virus.
[2] Numerator = number of infections, denominator = number observed; infections based on amnioallantoic or umbilical tumor and/or hemorrhagic lesions.
[3] Reed and Muench method of calculation.[20]

Table 8. Neutralization of Rous sarcoma virus; yolk sac inoculation method for detection.

Treatment[1]	Final RSV dilution			
	1:200		1:400	
	No. inf.[2]	Per cent inf.	No. inf.[2]	Per cent inf.
RSV + positive serum	0/13	0.0	0/7	0.0
RSV + negative serum	13/13	100.0	4/7	57.0
Positive serum control	0/14	0.0	0/8	0.0
Negative serum control	0/10	0.0	0/8	0.0
Simms' solution control[3]	0/9	0.0	0/8	0.0
RSV + Simms' control	11/12	91.7	5/8	62.0

[1] 10-day-old embryonated eggs were used; final dilution of serum 1:4; mixtures were held at refrigerator temperatures (4–7°C) overnight (12-15 hours).
[2] Infections were based on amnioallantoic or umbilical tumors, and/or hemorrhagic lesions. Hatched chicks were sacrificed and examined when 3 days old.

Since these titrations indicated that a relatively low dilution of serum of known RSV neutralizing capacity was required to neutralize relatively high concentrations of RSV, subsequent experiments on the feasibility of detecting neutralizing antibodies in chicken serum by yolk-sac inoculation of embryonated eggs were carried out with a 1:4 final dilution of serum (Table 8). Under the conditions of these experiments the respective RSV concentrations (1:200 and 1:400 final dilutions) were completely neutralized.

Influence of RVNA in hen sera on susceptibility of their eggs to RSV. Table 9 compares the activity of RSV in embryonated eggs from hens exhibiting RVNA with that in embryonated eggs from isolated control flocks. Differences were highly significant. These data were subjected to an analysis of variance and compared to the F-distribution ($F^{.05(1,10)} = 4.96$). Differences were highly significant (respective F-values between flocks 1 and 3, and 2 and 3, were 14.19 and 30.70). The variations between trials were also analyzed by the same method, and no significant differences were found ($F^{(5,8)} = 3.2$).

DISCUSSION

The effect of embryo age on nonspecific deaths indicated that the passage of the 20-gauge needle apparently caused irreparable damage to the very young embryo or to its poorly developed embryonic membranes. As the embryos aged, however, and the embryonic membranes became more highly developed, resistance to injury increased.

The hemorrhagic lesions observed during the later phases of embryonic development in embryonated eggs inoculated into the yolk sac with RSV were similar to those described by Duran-Reynals[24] and Lo and Bang.[38] These lesions were usually in the liver, occasionally in the heart and gizzard, and rarely in the subcutaneous tissues. Infected hatched chicks invariably exhibited neoplastic lesions at the periphery of the umbilical opening. To the authors' knowledge, this response has not been previously reported. The neoplastic response at this site appeared to be closely associated with remnants of the allantoic stalk.

Hemorrhagic lesions were almost always preceded by extensive amnioallantoic neoplasia. The relatively late appearance of hemorrhagic lesions indicated either that the action on endothelial cells resulting in hemorrhage was comparatively delayed, or that secondary focal lesions had occurred.

The earlier development of neoplastic lesions in 10-day-old embryonated eggs than in 8-day-old embryonated eggs may be explained by the increased folding of the epithelium of the yolk sac and the resultant increased rate of absorption of virus-containing yolk material by older embryos.[45] Total infections in 8-day-old embryonated eggs were comparable with 10-day-old embryonated eggs, however, when the postinoculation incubation times were extended 10 days or longer.

The inverse relation between age of fowl and susceptibility to RSV infection has been reported by many investigators.[18,19,20,52] Duran-Reynals and King[26] suggested that this resistance of the older fowl was linked with the presence of humoral factors. Prince[47] reported that variations in the resistance of embryonated eggs to RSV infection were not due to humoral neutralizing factors, since he was unable to detect

Table 9. Influence of Rous virus neutralizing antibodies (RVNA) in hens' sera on susceptibility of their eggs to Rous virus.

Flock no.	Age (mo.)	Sera reaction for RVNA	Log dilution ID_{50} of RSV[1]						Composite ID_{50}[2]
			1	2	3	4	5	6	
1	19-22	—	3.56	2.75	2.78	3.18	3.16	3.08	2.87
2	8-11	—	4.00		3.67		4.30	3.08	3.60
3	8-22	+	2.33	1.77	1.85	2.30	2.93	2.51	2.24

[1] Reciprocal of the logarithmic dilution of the ID_{50} of RSV calculated by the method of Reed and Muench.[20]

[2] Reciprocal of the logarithmic dilution of the composite ID_{50} calculated from totals of the separate trials by the method of Reed and Muench.[20]

an increase in RVNA with increasing age of the hens producing the eggs. He attributed the increased resistance to genetic factors. Carr[17] reported the presence of maternal antibody for RSV in the yolk of the egg, and since about 75% of the sera from hens of flock 3 (Table 9) showed RVNA activity, the results were not unexpected. The Rous neutralizing titer of the sera from flock 3 was not determined, however, and the respective 4-fold and 20-fold reductions in RSV activity noted below those of embryonated eggs from the isolated control flocks (Nos. 1 and 2) are only relative values. In the present study on the influence of the age of the hens producing the eggs (Table 5), it appears very improbable that RVNA or genetic factors were involved in the reduced susceptibility observed in the embryonated eggs from the older hens (flock 2), since: 1) the flocks were of the same genetic line; 2) flocks were held in isolation with precautions against exposure to RSV; and 3) sera from these hens were consistently negative for RVNA.

In the present studies, eggs from the RPRL Line 15 strain of White Leghorns were significantly more susceptible to RSV than either the commercial breeder or the WSU strains. The apparent influence of the genetic strain of the egg supply flock on RSV activity confirms reports by other investigators.[49,66,67]

Indirect evidence indicates that the yolk-sac method for the bioassay of RSV is considerably less sensitive than the intramuscular-intracutaneous methods,[44] the CAM pock-count

method,[64] or the tissue culture–infective center method,[57] but it is a comparatively simple, safe, and rapid procedure. Tests can be completed in about 10–14 days, compared with as long as 30 days for some of the other methods.

SUMMARY

The effect of Rous sarcoma virus (RSV) inoculated into the yolk sac of embryonated eggs and the influence of Rous virus neutralizing antibodies (RVNA) on RSV activity in the embryonated egg inoculated by the yolk-sac route were studied.

1) The number of deaths attributable to inoculation injury, or to causes other than Rous virus activity, depended on age of the embryonated egg, decreasing as age increased to 10 days.

2) A definite pathologic effect was produced in embryonated eggs when RSV was inoculated via the yolk sac. The earliest manifestation was a neoplastic proliferation of cells in the amnioallantois, followed by hemorrhagic lesions. Infected hatched chicks invariably exhibited hemorrhagic lesions on the liver and neoplastic lesions at the periphery of the umbilical opening.

3) Embryonated eggs appeared most susceptible to RSV infection when 10 days old.

4) When RSV was inoculated into the yolk sac of embryonated eggs, proliferative lesions on the amnioallantois were detected earlier in 10-day-old than in 8-day-old eggs.

5) The number of infections produced in embryonated eggs inoculated via the yolk sac was directly related to RSV concentration.

6) The age of the laying flock had a significant effect on the susceptibility of embryonated eggs to infection with RSV. This effect did not appear to be associated with transovarial passage of RVNA from hen to the egg, or with genetic factors.

7) The genetic strain of the egg source flock significantly influenced the activity of RSV in embryonated eggs inoculated via the yolk sac.

8) The yolk-sac method of inoculating embryonated eggs is relatively simple, safe, and rapid. Bioassays can be completed in about 10–14 days.

9) The concentration of serum containing RVNA required to neutralize RSV was directly proportional to the concentration of virus present, except that relatively high concentrations of serum were required to neutralize lower dilutions of RSV.

10) RVNA in hen sera definitely affected the susceptibility of their embryonated eggs to RSV.

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CASE REPORT: GENERALIZED GRANULOMATOUS LESIONS IN CHICKENS AND WILD DUCKS CAUSED BY ASPERGILLUS SPECIES*

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Introduction

ASPERGILLOSIS, a mycotic infection caused by various species of *Aspergillus*, is widely distributed throughout the world in a great variety of vertebrate hosts ranging from penguins[4] to alligators.[5] Several species of aspergilli grow freely in nature; *A. fumigatus*, common in the soil, is probably the most frequent offender in avian mycotic infections. The disease is not contagious, but the organism may be highly infectious, causing serious economic losses in chicks and turkey poults. Biester and Schwarte[1] provide an excellent review and extensive reference list of aspergillosis in birds.

Pulmonary granulomatous lesions are not uncommon in cases of aspergillosis, but the two cases reported here are unusual in that gross lesions consisted of extensive, generalized granulomata, with none of the typical yellow caseous nodules and plaques[2] usually observed, at least in the writer's experience, with *Aspergillus* infections. In neither case was aspergillosis suspected from gross lesions and symptomatology. Lesions were more suggestive of neoplasia or avian tuberculosis.

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CASE I

The first case, submitted to the Diagnostic Lab, Delaware Poultry Labs Incorporated, Millsboro, Delaware, in October of 1958, involved 10,000 broilers. Their history indicated that these 6-week-old birds were doing very poorly; they were extremely uneven in size, and though mortality was not excessively high, morbidity was estimated at about 30%. Signs of respiratory distress were not apparent.

Post-mortem examination of some of the affected birds revealed extensive granulomatous lesions of diameters ranging from 3–4 mm up to 4 cm. These nodules were unlike the typical ones seen with brooder pneumonia in that caseous cores were absent. The first impression was that the condition was one of malignant neoplasia with generalized metastasis. The high morbidity, however, did not support such a diagnosis. Cultures from some of the lesions, incubated at room temperature and at 37°C, revealed pure cultures of a mold not unlike *Aspergillus* species. Identification, by the U.S. Public Health Service, Chamblee, Georgia, revealed *A. flavus* sub-group *oryzae*. Histopathological studies of affected tissues revealed

Fig. 1. Granulomatous lesions in body cavity of Mallard duck.

granulomas with mycelial elements similar to those observed in typical cases of aspergillosis.

During the next three months several more cases of this same condition were presented to the laboratory. Morbidity varied from the 30% seen in this outbreak to less than 0.1%. All cases observed appeared over about a three-month period and were fed by the same commercial feed company. It is surmised that this particular feed company purchased a lot of grain heavily contaminated with the species of *Aspergillus* cited above.

The literature reveals that systemic infections of *Aspergillus* are rare. In this case it is significant that respiratory distress was not a common sign. Though gross pathology revealed many birds with numerous lesions in and about the lungs and air sacs, they were not numerous enough to cause apparent respiratory symptoms; the principal symptoms were cachexia and poor growth. Chute *et al.*[3] reported systemic fungus infections associated with surgical caponizing injuries in which there was no lung involvement but numerous granulomas in the skin and subcutis.

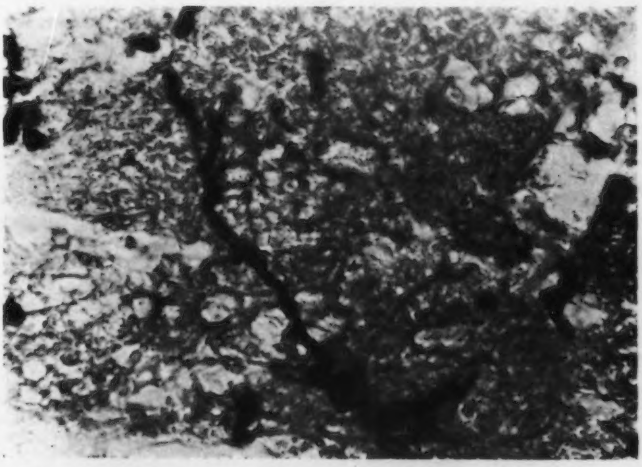

Fig. 2. Granulomatous lesion showing mycelial elements. ×900. Gridley fungus stain.

CASE II

The second case was in a wild Mallard duck shot on October 23, 1959, in Benton County, Washington. The carcass was submitted to the diagnostic laboratory at Washington State University. Post-mortem examination revealed multiple nodular lesions throughout the body cavity (Fig. 1). Because of the similarity of the gross pathology to that observed in a wild duck previously diagnosed as avian tuberculosis, this case was tentatively diagnosed as tuberculosis. Acid-fast staining of impression smears, and histological sections from several nodules, were nondiagnostic.

Ground tissue, including several nodules, was cultured on egg yolk agar and on Dubos oleic albumen agar. A mold resembling an *Aspergillus* species was isolated. Some of the same material was inoculated into four 4-week-old chickens, two intravenously and two intraperitoneally. These were TB-tested with avian tuberculin 8 weeks later, and all were negative. Post-mortem examination revealed no gross lesions. Histopathological studies of tissue sections of lesions from the duck stained by H & E and Hotchkiss McManus techniques

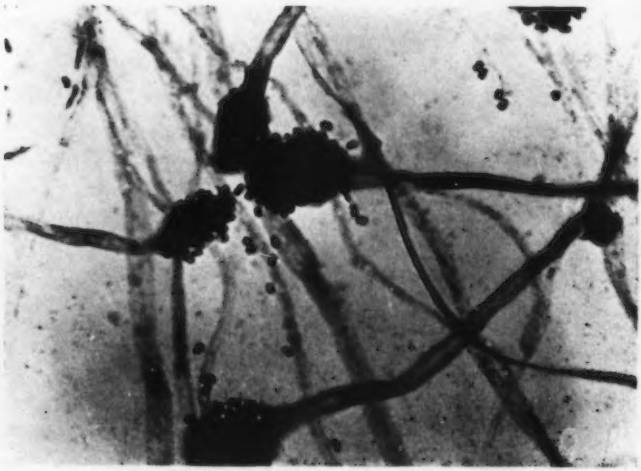

Fig. 3. Aspergillus fumigatus, cultural preparation. ×450.

revealed typical granulomatous nodules with numerous mycelial elements of branched septate hyphae (Fig. 2). Cultures on Sabouraud's dextrose agar were identified as *Aspergillus fumigatus* (Fig. 3) by Dr. L. M. Ringen, Department of Veterinary Microbiology, Washington State University.

SUMMARY

Two unusual cases of aspergillosis are reported. Case I involved 10,000 broilers suffering from an outbreak of a generalized mycotic infection caused by *Aspergillus flavus* sub-group *oryzae*. The second case, in a Mallard duck, was also a mycotic infection characterized by generalized granulomatous lesions. The etiological agent was *A. fumigatus*.

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EXPERIMENTAL PSITTACOSIS AND ORNITHOSIS IN TURKEYS. II. COMPARISON OF THE HISTOPATHOLOGY OF NINE STRAINS

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SINCE 1952, when natural infection of turkeys with a member of the psittacosis-lymphogranuloma group of agents was first proven,[6] the disease has been reported from many parts of the United States. The causative organisms have been identified by morphology, serology, and animal pathogenicitiy tests.[15,17] The results of these tests have been uniform except in pathogenicity for laboratory animals,[10,17] but there is much variation in the disease as seen in turkeys. The virulence of ornithosis in Texas,[7,18] Oregon,[9] and New Jersey[5] is in marked contrast with the rather mild symptoms and lack of mortality observed in Wisconsin,[12] California,[1] and Michigan.[13,14] Except for one outbreak in California,[1] ornithosis has occurred in human beings associated with the infected turkey.[12]

The histopathologic alterations produced in turkeys by one of the virulent strains[3] and one of the less virulent strains[11] have been described. Both strains produced similar lesions in the respiratory system and of the serous membranes, but the

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strain of low virulence apparently had no effect on the parenchymatous organs. This work was undertaken to study the comparative pathology produced in turkeys by various isolates of the psittacosis and ornithosis agent.

The gross pathology observed, described previously,[8] consisted of inflamation of the air sac, peritoneum, and pericardium; splenomegaly; and pneumonia.

PROCEDURE

Study was made of the histopathologic alterations produced in turkeys by nine isolates originally isolated from parakeets, parrots, or turkeys. The sources and identifying numbers of these isolates, and the agencies† making the isolation and identification were: *parakeet*, 6BC (Meyer), 56-69 (Davis); *parrot*, 56-68 (Davis); *turkey*, 57-5 (Pomeroy), 57-6 (Pomeroy), 56-57 (Dickinson), Ku (Davis), 56-253 (Davis), 56-237 (Davis).

The infectious agents were propagated in chicken embryos from a flock of hens that was serologically negative to tests for pleuropneumonia-like organisms. Amnioallantoic fluid, used as inoculum, was administered by the intratracheal route. The experimental birds were Broad Breasted Bronze turkey poults. Besides 5 birds maintained as uninfected controls, 90 three-week-old poults were divided into nine lots of ten each. Each lot was placed in isolation. Each of the experimental groups were inoculated with one of the isolates listed above. The procedure was repeated with 10- and 18-week-old turkeys, except that the 18-week-old group consisted of 10 groups of six birds each (only 60 birds were available).

After observation for two weeks surviving turkeys were killed. Autopsies were performed on all turkeys. Tissues were fixed in Zenker's solution. After embedding in paraffin, tissue sections were cut at 6 μ and stained by Mayer's hemalum and eosin and Wolbach's modification of Giemsa's stain. Selected tissues were stained by the Prussian blue, Gridley fungus, and Gridley reticulum methods.

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OBSERVATIONS

The lesions in the respiratory system consisted of tracheitis, pneumonitis, and fibrinous inflammation of the air sacs. Infiltrations of lymphocytes and granulocytes occurred in and beneath the mucosa of the tracha. Pneumonitis was common only in the 3- and 10-week-old birds. It was characterized by interstitial infiltrations of lymphocytes and mononuclear cells, combined with an exudate composed primarily of epithelioid cells filling the bronchial tree. The areas of respiratory tubules were cellular, apparently from proliferation of their lining cells and the presence of inflammatory cells. Lymphocytic foci were frequent, whereas exudation of large amounts of albuminous fluid was not. The lumina of tertiary bronchi often contained granulomatous foci. The air-sac membranes were thickened, and lymphocytic foci occurred beneath the epithelium in older turkeys. An exudate composed of fibrin, granulocytes, and mononuclear cells covered the respiratory surface of the membrane and often contained small granulomatous foci. Hyperplasia of the respiratory epithelium was indicated by the presence of folds and cystic spaces formed from the surface of the membrane.

The serous membranes, epicardium and peritoneum, were covered by a fibrinous exudate containing mononuclear cells, lymphocytes, and granulocytes that infiltrated the subserous tissue for some distance.

Hepatitis, splenitis, and myocarditis were common findings, especially in the experimental groups infected with turkey isolates. The hepatic sinusoids contained mononuclear cells and lymphocytes varying in distribution from small foci to diffuse infiltrations, and numerous lymphocytes were present in the portal areas. Kupffer's cells were conspicuous and engorged with an iron-positive pigment that was considered to be hemosiderin. The splenic lesions consisted primarily of reticuloendothelial proliferation combined with varying degrees of lymphoid proliferation, congestion, and hemosiderosis. In many of the younger turkeys dying from the infection, large areas of proliferated reticuloendothelial cells had undergone necrosis. Myocarditis was common, consisting of infil-

Table 1. The occurrence of microscopic lesions

Source and agent	Number of turkeys[1] with:						
	Trache-itis	Pneu-monitis	Inflam-mation of air sac	Peri-carditis	Myo-carditis	Hepa-titis	Splen-itis
Parakeet							
6BC	6	2	14	3			
56-69	15	4	22	6	1		
Parrot							
56-68	14	5	18	8	2	3	3
Turkey							
57-6	14	6	16	10			
57-5	15	7	20	17	6		1
56-57	8	9	19	20	17	19	14
Ku	10	9	21	22	11	20	19
56-253	8	12	13	18	19	18	15
56-237	17	16	23	24	21	24	24

[1] Of 26 infected with each agent. There were 15 control birds, and none showed lesions.

trations of mononuclear cells and lympohcytes between the muscle fibers. These foci were present in both the superficial and deep layers of the myocardium.

The occurence of lesions is shown in Table 1. All isolates produced lesions in the respiratory system and serous membranes, but alterations in the parenchymatous organs were much more common and severe in groups inoculated with isolates 56-57, Ku, 56-253, and 56-237, all originally from turkeys with the more virulent form of ornithosis. Lesions of the parenchymatous organs were rarely produced with the parakeet isolates, and not much oftener with the parrot and mild turkey isolates. Myocarditis, when present in turkeys in these groups, occurred only in the superficial layers of the myocardium and appeared to be an extension of infection from the pericardium.

Typical elementary bodies were observed in macrophages in the lung, air sac, pericardium, liver, and spleen in the Giemsa-stained sections (Fig. 1). These bodies were observed most frequently in younger turkeys in the groups infected with the virulent turkey isolates. They were identified in nine birds infected with 56-37, five infected with Ku, nine infected with 56-253, and thirteen infected with 56-237. Elementary bodies of unequivocal nature were rare in tissues in the

other groups and were not identified in tissues from the uninfected controls.

DISCUSSION

The lesions observed in this study were similar to those produced in turkeys by the Jo strain of ornithosis[3] and to those in turkeys infected with one of the less virulent turkey strains.[11] Except for a tendency of the psittacine isolates and the avirulent turkey strains to remain "localized" in the respiratory system and serous membranes, the lesions of ornithosis and psittacosis vary only in incidence and severity. In effect on the respiratory system all nine of these agents were similar and nearly equal, whereas in ability to produce sys-

Fig. 1. Cluster of elementary bodies in the cytoplasm of a macrophage *(arrow)* in the pericardium of a turkey infected with a virulent strain of the ornithosis agent. Giemsa, × 2,400.

temic disease the turkey strains of low virulence seem more related to the psittacine isolates than to the virulent turkey isolates. Considering their indistinguishable pathogenesis, it is interesting to speculate on the effect that serial passage in turkeys might have on the virulence of mild turkey strains and psittacine isolates.

The symptomatology, mortality, and incidence of lesions in groups infected with isolate 56-237 agree closely with that observed in turkeys infected with Jo ornithosis. Since both of these agents were originally from turkey flocks in the same geographical area in South Texas, it may be illogical to regard them as separate "strains" of ornithosis.

Elementary bodies have been observed in most of the species in which psittacosis-ornithosis has been studied, but they have not been demonstrated in each individual animal infected.[19,20] The infrequency with which unequovical elementary bodies were identified in the tissues from the groups infected with 57-5 and 57-6 is not explained. Other workers have observed them in large numbers in both the tissues and exudates from turkeys naturally and experimentally infected with avirulent turkey strains.[1,11]

Since the gross and microscopic lesions of PPLO (pleuropneumonia-like organisms) and ornithosis infections in turkeys possess several features in common, the problem of diagnosis must be considered. Differentiation of these diseases by histopathological means is not complicated when only the virulent turkey strains of the ornithosis agent are involved, since the lesions of PPLO infections are not systemic.[4] However, the lesions of both PPLO and avirulent ornithosis are limited to the respiratory system and serous membranes, and alterations of similar type occur in the air sacs. Although the characteristic viral pneumonitis of ornithosis contrasts with the bacterial type of pneumonia produced by PPLO,[4] pneumonitis is rarely observed in turkeys after the age of 18 weeks. Pericarditis is rare in turkeys infected with PPLO.[21] The presence of typical elementary bodies is diagnostic for ornithosis.

No evidence of sinusitis was observed in any of the control

birds or in the supply flock from which these turkeys were obtained. No bacterial or PPLO isolations were made on the birds used in this study, since in most experimental ornithosis and PPLO infections in turkeys the pericardial and air sac exudates have been found to be bacteriologically sterile.[2]

SUMMARY

The lesions produced in turkeys by nine isolates of the psittacosis-ornithosis agent are described. Alterations of similar type were noted in the respiratory system and the serous membranes in all groups. In addition, the virulent turkey strains of the agent also produced systemic disease, as evidenced by myocarditis, hepatitis, and splenitis. With few exceptions the lesions of the psittacine isolates and avirulent turkey strains were limited to the respiratory system and serous membranes. Elementary bodies were numerous in tissues of birds infected with the virulent turkey strains, and rarely observed in birds infected with the psittacine isolates and avirulent turkey strains.

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ANTIGENIC AND IMMUNOLOGICAL STUDIES ON SEVERAL ISOLATES OF AVIAN INFECTIOUS BRONCHITIS VIRUS*

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A PREVIOUS communication[2] reported studies on 14 isolates of infectious bronchitis virus (IBV) by reciprocal serum neutralization (SN) tests. Three isolates were found antigenically different from the usual pathogenic strains of IBV and different from each other as determined by SN tests. The present study deals with an additional 5 isolates of IBV, including two commercial vaccine strains. The serological relationship of these isolates and the three antigenically different isolates mentioned above were studied by SN tests. Cross-immunity studies were also done to correlate SN with cross-challenge results.

MATERIALS AND METHODS

Isolates 609, 97, 704, 581, and 661 were isolated at Veterinary Medical Research Institute from chickens with acute respiratory symptoms; 33 was isolated at the New York State Veterinary College; 730 and 758 were isolated from commercial infectious bronchitis vaccines; 726 and 727 were received from Dr. L. C. Raggi, University of California; A5968, received from Dr. Roy Luginbuhl, University of Connecticut, is commonly referred to as the Connecticut or L-2 strain. Three antiserums had been prepared for a previous study.

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Table 1. Serum neutralization tests using 5 virus isolates and 8 antiserums.

Virus	Antiserums							
	730	758	704	726	727	97	609	5968
730	5.4[1]	4.2	2.2	3.0	3.4	2.0	1.3	1.3
758	3.9	2.2	2.5	3.0	2.7	1.3	1.5	1.2
704	4.2	3.7	6.2	4.1	3.2	1.2	1.2	1.3
726	4.1	5.3	2.8	6.4	4.0	1.0	1.3	1.5
727	5.4	4.2	3.3	3.4	6.7	1.2	1.2	1.4

[1] Log. neutralizing doses.

Five additional antiserums were prepared by exposing chickens to an aerosol of the virus, followed in 2–3 weeks by an intramuscular inoculation of 5–10 ml of infective allantoic fluid. Blood was collected 10–14 days later. The SN tests were performed according to Cunningham,[1] modified as described preously.[2]

PROCEDURES AND RESULTS

Serum neutralization tests were performed with the 8 antiserums against 5 virus isolates (Table 1). The neutralizing indexes varied considerably among 730, 758, 704, 727, and 727. Except with the 758 antiserum, there was a strong neutralization of the homologous virus. Antiserums from 609, 97, and 5968 were not capable of neutralizing any of the other virus isolates. In an attempt to corroborate the SN results by cross-challenge tests, the following trials were set up:

Trial 1. Five groups of 6–7 chicks were housed in modified Horsfall units, and each group was inoculated by aerosol with a different isolate of bronchitis virus. An additional group served as uninoculated controls. After recovery all were challenged by aerosol with a typical pathogenic isolate (581) of IBV (Table 2). Chickens recovering from infection with 609 and 97 apparently had some degree of resistance, whereas no resistance was demonstrated in chickens recovering from 5968. Recovery from infection with 661 and 33 resulted in complete resistance to the challenge virus.

Trial 2. Following recovery from an aerosol exposure to 704 bronchitis virus, 43 chicks were divided into small groups and challenged with 5 heterologous isolates and the homol-

Table 2. Aerosol challenge tests following recovery from 5 different bronchitis isolates.

Infection with isolate no.	Duration (days) of symptoms	Symptoms after challenge with 581 isolate
5968	10	7 of 7[1]
609	7	4 of 7
97	8	3 of 7
661	11	0 of 6
33	14	0 of 7
Controls	none	6 of 6

[1] Number with symptoms of number challenged.

ogous isolate (Table 3). There was evidence of only a partial immunity to 97, 609, and 5968, but a solid immunity to the other isolates. A satisfactory challenge was not obtained with 609, because only 2 of 4 controls developed symptoms.

Trial 3. Seventy-one chickens that recovered from an aerosol of 609 were divided into small groups for challenge (Table 3). Challenge with 727 and 730 resulted in some infection. However, there was a fairly good immunity to the other challenge isolates. SN tests were done on 4 pools of serum collected from blood samples taken by heart puncture just before challenge. Each serum pool consisted of serum from 2 birds. The results (Table 4) indicate that the birds should have been susceptible to all the challenge isolates, since the serums failed to neutralize any of the isolates to any significant degree. The neutralizing index with the homologous virus (609) was not as high as desirable.

Table 3. Aerosol challenge of chickens with heterologous bronchitis isolates following recovery from infection.

Challenge with virus isolate	Recovered from infection with bronchitis isolates:					
	704	609	97	5968	727	Controls[3]
97	5/6[1]	1/12	—[2]	0/12	0/9	13/13
5968	5/13	2/12	0/18	—	0/9	20/21
758	0/6	1/11	0/22	1/13	0/9	17/17
609	1/6	—	0/18	1/12	0/9	14/16
727	0/6	12/12	10/18	9/12	—	16/16
704	0/6	0/12	0/20	1/12	0/9	15/16
730	—	5/12	13/22	7/13	0/9	13/13

[1] Means 5 of 6 had symptoms of tracheal rales after challenge.
[2] — means not done.
[3] Combined results of 5 different challenge experiments.

Table 4. Average serum neutralization index with serum pools from chickens recovered from infection with bronchitis isolates.

Virus isolate	Serum			
	609	97	5968	727
97	1.0	6.9	2.0	1.4
5968	1.6	1.2	4.6	1.3
758	not done	not done	1.5	4.6
609	3.8	2.0	1.1	1.5
727	2.5	1.8	2.0	5.9
704	1.2	2.0	1.2	4.4
730	1.2	2.7	1.8	3.5

Trial 4. One hundred and twelve 18-day-old chicks were exposed to an aerosol of 97. Eighteen days later they were again exposed to an aerosol of the same material. About 5 weeks after the original exposure the birds were divided into groups and challenged with 6 heterologous isolates (Table 3). The chickens were susceptible to two isolates–727 and 730–but resistant to the other 4 isolates. Serum pools collected at challenge were used in the SN test against the challenge isolates. Again it was found that neutralizing indexes were low against all the isolates except the homologous isolate 97. These results are in Table 4.

Trial 5. Seventy-four chickens 5 weeks old were exposed to 5968 by aerosol. Twenty-six days later the birds were divided into groups and challenged with 6 heterologous isolates (Table 3). The birds were susceptible only to 727 and 730; they were essentially resistant to the other four. Serum pools collected at challenge were used in SN tests (Table 4). None of the serum pools was capable of neutralizing the virus isolates to any satisfactory degree. The average neutralizing index against the homologous isolate was lower than usual with this strain; perhaps collection of serum was early.

Trial 6. Fifty-four chickens 5 weeks old were inoculated by aerosol with 727 IBV in the 7th embryo passage. Marked respiratory symptoms persisted for a week. Five weeks later the birds were challenged by aerosol with 6 isolates. The birds were resistant to all the challenge isolates (Table 3). Serum collected at challenge neutralized 704, 758, 730, and the homol-

ogous isolates to varying degrees, but was unable to neutralize 97, 609, 5968 (Table 4).

DISCUSSION

Raggi and Lee[3] found some discrepancy between the serum neutralization and challenge test results in their study of the relationship between immunity and serum neutralization titers. In our studies too, the results of serum neutralization tests and cross-challenge tests were far from agreement. There appears to be some cross-immunity between all these strains, although the serum neutralization tests do not demonstrate this. The common pathogenic virus isolates, such as 727, 730, 758, and 704, were more effective in immunizing against all the isolates than were the three isolates 97, 609, and 5968. The most pathogenic isolate, 727, stimulated the best immunity to all strains. Isolates 727 and 730 were most capable of overcoming immunity following recovery from infection with either 609, 97, or 5968. It appears that tissue immunity of the respiratory tract mucosa is independent of serum antibodies. How long this tissue immunity persists is not known. In one trial, not reported here, chickens 11 weeks after recovery from infection with 97 were immune to an aerosol challenge of several strains even though they had no demonstrable serum antibody levels against the challenge strains.

SUMMARY

Isolates of avian infectious bronchitis virus were used to infect 6 groups of young chickens. Following recovery the chickens were divided into small groups and challenged by aerosols of heterologous isolates of bronchitis virus. Serum neutralization tests were also done. The cross-challenge results did not agree with the serum neutralization results. There appeared to be some cross-immunity between the several isolates of virus used, including 609, 97, and 5968, which had previously been found antigenically different by reciprocal serum neutralization tests. Tissue immunity of the respiratory tract mucosa appears to be an important factor in resistance

to challenge following recovery from infection with infectious bronchitis virus.

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ACID AND ALKALINE PHOSPHATASE LEVELS IN COCCIDIOSIS-INFECTED CHICKENS

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THE phosphatases are enzymes responsible for many functions in the body metabolism. The functions studied most extensively are concerned with: 1) absorption of food from the gastrointestinal tract, 2) dephosphorylation within the liver, 3) reabsorption of glucose by the renal tubules, and 4) deposition of lime salts within the bones.

Many pathological conditions reflect increased levels of acid and alkaline phosphatases. Alkaline phosphatase values of clinical diagnostic significance have been noted in diseases such as osteogenic sarcoma, osteitis fibroma cystica, rickets, and osteomalacia.[12]

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It has been recorded that certain nutritional states influence the serum alkaline phosphatase levels in chickens. It was found[17] that a diet deficient in magnesium produced a low level. It was also discovered that a diet deficient in Vitamin D[9,10,11] and one with excess fluorine each produced a low serum alkaline phosphatase. A high-fat diet caused an increase of serum alkaline phosphatase due to increased fat absorption.[15] Attempts [14,16] have been made to relate the activity of this enzyme to genetics.

Some workers[6] reported serum alkaline phosphatase to be greater in high-producing hens than in poor-producing hens. Recently[16] serum alkaline phosphatase was found to be higher in Leghorn chickens, 2–26 weeks old, than in a random-bred line. They also found a close relationship of enzyme activity levels between growth rate and age. At hatching there were no differences due to line or sex. The enzyme has been reported[1,2,3,14] to be higher in adult than in growing chickens, and higher in the laying hen than in the adult male.

Low alkaline phosphatase levels were observed in serum from birds with visceral lymphomatosis.[7]

One recent study[8] indicated the value level of plasma alkaline phosphatase declined between the ages of five and seven weeks, increased at the ages of eight and ten weeks, and declined to a very low level at the age of six months.

Workers[4] in India described the alkaline and acid phosphatase activity in the intermediate stages of *Eimeria tenella* infections in the cecal walls of White Leghorn chicks. They found that the gradation of reactions of phosphatase is directly proportional to the reaction for nucleic acid and the tempo of physiological activity of *E. tenella*. They found strong alkaline phosphatase activity at the micropyle of the oocyst and demonstrated the constant presence of phosphatases in the nucleus of the parasite. Acid phosphatase and 5-nucleotidase were present in the karyosome at all stages of the coccidium.

A preliminary study[18] in conjunction with a coccidiosis species survey in Maine indicated that birds undergoing active infection with coccidiosis have lower serum and tissue alkaline

phosphatase than birds already immune to the disease. This finding encouraged the authors to do the study reported in this paper.

MATERIALS AND METHODS

A group of 30 White Leghorn chickens, five weeks old, hatched at the Department of Animal Pathology, University of Maine, and reared disease-free in isolation in wire-floor cages, was divided into two groups. Twenty birds were placed in a cage for challenge with coccidia. Ten were segregated as controls.

They were maintained on a regular broiler pelleted feed with a continuous water supply.

The twenty birds were challenged with a quantity of oocysts known to produce intestinal coccidiosis uniformly. (The challenge material, containing *Eimeria maxima*, *E. necatrix*, and *E. acervulina*, was provided by courtesy of Sterwin Chemical Co., Inc., New York, and received from S. A. Edgar, Alabama Polytechnic Institute, Auburn.) The birds were challenged with 1 ml of the coccidia through a long glass pipette inserted in the esophagus as far as possible. The controls were kept free of the disease.

All challenge and control birds were necropsied five days after challenge. Before necropsy the birds were bled from the heart, and plasma was removed from the heparinized sample.

Four sections of intestine of uniform size were cut from each of three areas: the loop of the duodenum, the small intestine just anterior to the yolk stalk, and the colon anterior to the ceca. The tissues were placed in individual containers, and identified as to source. One piece of tissue from each area was placed in formolsaline for histopathological studies, one piece was put in cold acetone for enzyme studies, one was used for dry-weight determination, and one section was placed in distilled water.

Serum alkaline phosphatase determination was with the following technique: one ml of alkaline-buffered substrate (commercial preparation from Sigma Chemical Co.) was pipetted into each of two spectrophotometer (Klett) tubes.

They were placed in a 38°C constant-temperature water bath until temperature equilibrium was reached (about 5 min). Then 0.1 ml of water was pipetted into one control blank tube and 0.1 ml of serum into the other. The tubes were placed in a 38°C water bath and, 30 minutes later, 10 ml of 0.02*N* NaOH was added to each tube. The blank was placed in the Klett-Summerson colorimeter, and the instrument was set to 0 optical density (OD) using a wavelength filter of 400–420 mμ. No correction was made for color of the plasma since diluting the plasma 1:10 with H_2O made its OD negligible. The alkaline phosphatase activity of the serum was determined from the calibration curve of the instrument as described in Sigma Technical Bulletin No. 104.[13]

The tissue studies were carried out in the same manner except that the tissues were homogenized, weighed, and diluted, 1 μg to 1 ml of water. The dry weight was calculated as percentage of wet weight, and on that basis the activity rates per gram of dry tissue were determined. These calculations gave the activity rates of the dry tissue at the rate of 1 g per hour.

Histochemical examination for the presence of the enzyme was carried out according to Gomori's method.[5] This procedure is based on the principle that if sections are incubated with glycerophosphate in an alkaline reaction in the presence of Ca ions, the phosphate ions liberated will be precipitated as insoluble Ca phosphate. The latter is changed in a second step into metallic silver or black cobalt sulfate. In the histochemical staining procedure the sections of intestine were placed in a 37°C incubator in a mixture containing the sodium glycerophosphate and incubated for one hour. The tissues were stored in a refrigerator at 4°C until examination.

EXPERIMENTAL RESULTS

Four birds died from the challenge group, leaving only sixteen for study. All controls lived. The challenged birds had ruffled plumage and bloody feces. Gross lesions of a severe catarrhal to hemorrhagic enteritis throughout the intestine included masses of blood and oocysts in the ceca.

Table 1. Average phosphatase levels of serum and sections of intestinal tract of coccidiosis-infected chickens.

	Alkaline phosphatase		Acid phosphatase	
Tissue	Infected	Controls	Infected	Controls
Serum	7.54[1]	55.41**	5.83	3.41**
Upper intestine	7.02[2]	6.98**	0.541	0.6963
Middle intestine	0.365	0.898*	0.269	0.5487**
Lower intestine	1.52	0.477	0.583	0.341

[1] Bessey-Lowery units expressed as m*M* of nitrophenol liberated per liter of serum per hour.
[2] Modified Bessey-Lowery units expressed as m*M* of nitrophenol liberated per gram of dry tissue per liter of water per hour.
** Significant at the 1% level.
* Significant at the 5% level.

Microscopic studies showed many oocysts and intermediate forms from all portions of the intestinal tract. Histological evidence confirmed the presence of the disease.

The histopathology did show that the lower portion of the intestine just anterior to the ceca had the fewest intermediate stages of coccidia and intestinal damage.

Table 1 shows the average phosphatase levels found in the infected and normal control chickens. This table particularly shows the values of the uninfected, control, six-week-old chickens. No outstanding differences were noted between infected and control birds in acid phosphatase level. There was an extreme difference between infected and normal chickens in alkaline phosphatase serum values, however.

Table 2 presents statistical evaluation of the study. Differences were significant between infected and control birds in alkaline phosphatase values in the serum and the upper and middle gut. In fact, the serum and upper-gut differences were significant at the 1 % level. There was no significant difference in the lower-gut values. This table also shows that the acid phosphatase values were significantly different at the 1% level in the serum and middle gut, and insignificant in the upper and lower gut.

Table 3 summarizes the microscopic studies. Gomori's stain shows alkaline phosphatase as a black stain. Although the various stages of coccidia stained black, there usually was also a heavy black portion at the periphery of the epithelium

Table 2. Statistical evaluation of phosphatase levels of coccidiosis-infected chickens.

Material	Alkaline phosphatase				found t	Acid phosphatase				found t
	Infected birds		Control birds			Infected birds		Control birds		
	Av.±s.e.[1]	Range	Av.±s.e.	Range		Av.±s.e.	Range	Av.±s.e.	Range	
Serum[2]	7.5±1.3	1.3–17.0	55.4±6.3	21.5–96.0	7.515**	0.08±0.01	0.001–0.235	0.27±0.02	0.16–0.40	6.981**
Upper Gut[3]	3.3±0.8	0. –11.4	7.0±0.3	2.3–12.6	3.041**	0.54±0.02	0.11 –1.37	0.70±0.13	0.01–1.205	1.047
Middle Gut	0.4±0.1	0. – 2.1	0.9±0.2	0.1– 2.2	2.249*	0.27±0.04	0. –0.65	0.55± .07	0.20–1.18	10.769**
Lower Gut	1.5±0.6	0. – 7.8	0.5±0.1	0. – 1.2	1.667	0.58±0.17	0.16 –2.77	0.34± .03	0.11–0.59	1.367

[1] Average ± standard error.
[2] Bessey-Lowery units expressed as m*M* nitrophenol liberated per liter of serum per hour.
[3] Modified Bessey-Lowery units expressed as m*M* of nitrophenol liberated per gram of dry tissue per liter of water per hour.
* Value at 5% level, 2.064.
** Value at 1% level, 2.797.

Table 3. Histological alkaline phosphatase results[1] in 3 levels of intestinal tissue of chickens infected with coccidiosis.

Bird no. infected	Upper		Middle		Lower	
	H[2]	H & E[3]	H	H & E	H	H & E
1	2+	2+	2+	3+	2+	1+
2	2+	3+	1+	3+	2+	1+
3	2+	3+	2+	3+	2+	3+
4	3+	3+	2+	3+	3+	1+
5	3+	2+	2+	3+	2+	1+
6	2+	3+	2+	3+	2+	2+
7	1+	2+	2+	2+	1+	1+
8	1+	3+	1+	3+	1+	1+
9	1+	3+	1+	3+	2+	1+
10	4+	3+	4+	2+	4+	1+
11	3+	3+	1+	2+	2+	1+
12	1+	3+	2+	2+	1+	1+
13	2+	2+	1+	2+	3+	1+
14	2+	3+	2+	3+	3+	2+
15	2+	1+	1+	3+	1+	1+
16	2+	2+	1+	3+	2+	1+
Controls						
17	2+	—	2+	—	—	—
18	3+	—	2+	—	—	—
19	3+	—	—	—	—	—
20	1+	—	—	—	—	—
21	2+	—	—	—	—	—
22	4+	—	2+	—	—	—
23	2+	—	—	—	—	—
24	2+	—	—	—	—	—
25	2+	—	2+	—	—	—
26	4+	—	4+	—	3+	—

[1] + Slight; ++ moderate; +++ extensive.
[2] Gomori's histochemical stain for alkaline phosphatase.
[3] Hematoxylin and eosin stain.

of the villi. The microscopic evidence generally substantiated the biochemical analysis in that the lower intestine contained the fewest coccidia and the least intestinal damage.

DISCUSSION

It is not presumed that these studies will simplify a diagnosis of intestinal coccidiosis in chickens. However, since phosphatases are known to be involved with absorption of food from the gastrointestinal tract and if coccidia contain considerable phosphatase,[4] then in such an infection these levels may be altered. Another theory is that the intestinal destruction by the coccidia and subsequent hemorrhage released large quantities of the enzyme in the intestine. This

may not be reabsorbed, but is passed out in the feces. This loss of phosphatases affects fat utilization, altering the nutritional status of the bird.

Both gross and microscopic examinations revealed the least infection in the lower intestine, and this was supported by the biochemical values obtained.

The range of values was wide. For instance, as to the alkaline phosphatase values: for the upper gut there was only one infected bird with a zero value, for the middle gut there were seven birds with a zero value, and for the lower gut there were five birds with a zero value. However, since the range was not wider than that found, perhaps a more critical method of evaluation would be in order.

A careful study of the acid phosphatase group revealed the upper-gut group to have a very narow range between the infected birds and controls. The middle-gut portion was significant at the 1% level, and it was noted that the values were very close, eight of 16 being from .20 to .40 Modified Bessey-Lowery Units.[13] The lower gut had a wide range of values, but eight of the infected birds were from .20 to .50 Modified Bessey-Lowery Units.

The control of coccidiosis is a continuing problem. Sanitation has been helpful but is not a practical solution. Coccidiostats have been used for many years, with varying degrees of success, but these have not been consistent. Possibly, some of the failures are explained by the development of resistant strains of coccidia. New species of coccidia are being discovered that have not been tested for susceptibility to coccidiostats. Finally, the duration of immunity has not been determined for many of the species. It is possible that a given exposure, either natural or artificial, will not immunize for the bird's life. Consequently, much research still needs to be done before a completely satisfactory solution is found to this major poultry disease problem. Perhaps this contribution will stimulate other histochemical studies.

SUMMARY

Histochemical studies revealed distinct differences in alkaline phosphatase between coccidiosis-infected and unin-

fected chickens. Biochemical values showed statistically significant differences in both plasma acid and alkaline phosphostase levels between infected and uninfected chickens. Alkaline phosphatase levels were statistically significant in upper and middle gut but not the lower gut of infected vs. uninfected chickens. Acid phosphatase levels were statistically significant in the middle gut but not in the upper or lower gut of infected vs. uninfected chickens. There was some correlation between microscopic evidence of infection, histochemical staining for alkaline phosphatase, and biochemical values.

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MULTIPLICATION AND CYTOPATHOGENICITY OF EASTERN ENCEPHALITIS VIRUS IN A DUCKLING KIDNEY CELL CULTURE SYSTEM*

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EASTERN encephalitis virus (EEV) has been propagated in various cell culture systems.[1,2,4,6,8,9,10] The isolation of EEV from ducklings[3] prompted an attempt to propagate the virus in a duckling kidney cell cutlture system. The results are presented herein.

MATERIALS AND METHODS

Monolayer cultures of duckling kidney cells in tubes were prepared employing a technique similar to that for the preparation of chick kidney cell cultures[5] except that 20 ml of trypsin solution was used for each pair of kidneys, and cells obtained from each pair of kidneys were suspended in 300 ml of medium containing 5% calf serum. Monolayer cultures of continuous cell sheet were inoculated with 0.1 ml of seed virus in 0.9 ml of maintenance medium per culture and incubated at 36 C.

* From the Duck Disease Research Laboratory, Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Eastport, Long Island, New York.

The seed virus, originally isolated from ducklings, was contained in yolk material representing the eighth chicken embryo passage. Medium 199[7] was used as maintenance. Serial passage of the virus was made every 24 hours through ten passages. Cultures were examined microscopically twice each day for evidence of cytopathogenic changes.

Parallel titrations of the seed virus and the cell-culture tenth-passage virus were performed in six-day-old chicken embryos by inoculating 0.2 ml of the virus dilutions into the yolk sac and 0.1 ml in the cell culture system. The cell-culture tenth-passage virus was identified by neutralization tests performed in the cell culture system using a 1:1 dilution in maintenance medium of immune serum from the horse (received from Dr. D. Stamm, CDC, Montgomery, Alabama), duck and chicken. Both titration and neutralization tests were read daily for three days post-inoculation.

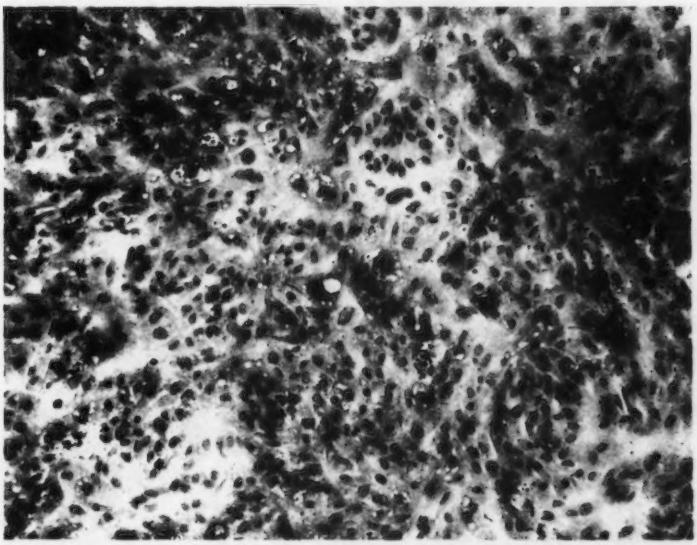

Fig. 1. Control culture, harvested after 12 hours' incubation in maintenance medium, showing a continuous sheet of integrate cells. H. & E. stain; × 100.

RESULTS

The control cultures were seen as a continuous sheet of integrate cells (Fig. 1). The cytopathogenic changes in infected cultures were enlargement and increase of refractibility of the nuclei, followed by cytoplasmolysis. At the height of the changes there were multiple holes in the cell sheet and the culture was composed mainly of partially or completely naked nuclei with cytoplasmic threads and debris (Fig. 2). Practically no cells remained on glass after 24 hours' incubation. Destruction of the inoculated cultures was observed in all passages.

The titer of the seed virus was $10^{-7.4}$/0.2 ml in chicken embryos and $10^{-6.3}$/0.1 ml in the cell culture system. The cell-culture tenth-passage virus titrated $10^{-5.0}$/0.2 ml in chicken embryos and $10^{-5.5}$/0.1 ml in the cell culture system. The cytopathogenicity of the cell-culture-passage virus was com-

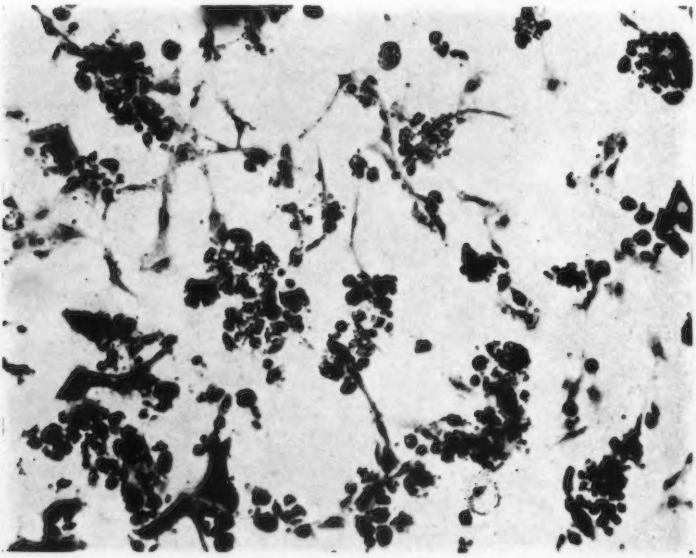

Fig. 2. Inoculated culture, harvested 12 hours post-inoculation. Advanced cytoplasmolysis rendered numerous nuclei naked or with only scanty cytoplasmic threads and debris. H & E stain; ×100.

Table 1. Results of neutralization tests using the cell-culture tenth-passage eastern encephalitis virus and normal or immune sera in the duck embryo kidney cell culture system.

Sera	$CCID_{50}$ * of 10th passage EEV neutralized
Normal horse #1	6
Normal horse #2	10
Immune horse	$\geq$ 200,000
Normal duck #1	10
Normal duck #2	32
Immune duck	$\geq$ 200,000
Normal chicken #1	40
Normal chicken #2	4
Immune chicken	$\geq$ 200,000

* Cell-culture infective doses (virus titer was $10^{-5.0}$).

pletely neutralized by the 1:1 dilution of the immune sera, but not by the diluted normal sera (Table 1).

DISCUSSION

The cell-culture tenth-passage virus was completely neutralized by homologous EE immune sera, as evidenced by the absence of the cytopathogenic effect. Normal serum did not have this action. These results indicated that EEV was the agent originally inoculated and that the cytopathogenic changes observed in the virus-inoculated cultures were specific. The infectivity of the cell-culture-passage virus was relatively high for chicken embryos and cultured duckling kidney cells. It was comparable to that of the original seed virus after ten passages in the cell culture system. The maintenance of high infectivity, together with the definite cytopathogenic changes induced in the cultures by the inoculated virus, justifies the conclusion that the virus had multiplied in the system.

SUMMARY

Eastern encephalitis virus of duckling origin was carried for ten passages in a duckling kidney cell culture system. Cyto-

pathogenic changes were observed in all ten passages. The cell-culture tenth-passage virus was identified by neutralization tests. The comparative titers of the seed virus and the cell-culture tenth-passage virus in chicken embryos and in the cell culture system, as well as the results of virus neutralization tests, are presented.

ADDENDUM

After this manuscript had been submitted for publication, the cytopathogenicity of, and plaque formation by, three strains of EEV (Pheasant, Belem, and standard) in Pekin duck kidney cell cultures were reported (Henderson, J. R. and R. M. Taylor. J. Immunol, 84:509. 1960).

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ELECTRON MICROSCOPIC SURVEY OF THIRTEEN STRAINS OF *MIYAGAWANELLA**

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Received 25 October 1960

SINCE photomicrographs of psittacosis-pneumonitis-lymphogranuloma venereum organisms of the genus *Miyagawanella*[5] appear frequently in the literature without comparative photographs of other strains classified in the genus, an electron micrograph survey was undertaken of a variety of strains in the Miyagawanella collection of the University of

Table 1. Source of Miyagawanella strains observed under the electron microscope.

Class	Number	Specific host	Strain code	Investigated or isolated by
Avian	1	Parakeet	X1	Hooper Foundation (1955)*
	2	Parakeet	Pensacola	Hooper Foundation (1955)*
	3	Parakeet	Robinson	Hooper Foundation (1954)*
	4	Dove	Dove 33	Hooper Foundation (1951)*
	5	Chicken	Friend	Karrer *et al*[8]
	6	Turkey	C-1	Bankowski and Page[2]
	7	Turkey	NJ-1	Page[12]
	8	Turkey	NJ-4	Page[13]
	9	Egret	SE-45	Rubin *et al.*[15]
Human	10	Man	Borg	Olson and Larson[11]
	11	Man (?)	MP	Francis and Magill[6]
Mammalian	12	Cat	FP	Baker[1]
	13	Cow	EBA-59-795**	Storz *et al*[16]

* These Miyagawanella strains are organisms recovered from field cases submitted for diagnosis to the George Williams Hooper Foundation for Medical Research, University of California Medical Center, San Francisco, and examined by various members of the Hooper Foundation staff. The year of submission is listed in parenthesis.

** Isolated from a cow with epizootic bovine abortion (EBA).

* This investigation was supported in part by a grant from the American Cyanamid Co. (Lederle Division) and by U. S. Public Health Service grant number 5868.

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California. The purpose was to compare the structural features of strains derived from avian and mammalian (including human) hosts and prepared and photographed under similar conditions. Many excellent micrographs of individual strains have been published.[4,7,14,17,18] Three are included for comparison (Figs. 44 to 46), by permission of the authors and publishers.

MATERIALS AND METHODS

Source of strains. Strains numbered 1, 2, 6, 7, and 8 in Table 1 were isolated by the senior author from various avian species. Strains 3, 4, 5, 9, 10, and 12 were obtained from Dr. Bernice U. Eddie, University of California Medical Center, San Francisco, and strain number 11 was obtained from Dr. T. T. Crocker, also at the Medical Center. Strain number 13 was isolated by the second author at the University of California, Davis.[16] All of the strains were maintained by propagation of infectious material in the yolk sac of embryonating chicken eggs followed by storage of infected yolk sacs in a dry-ice chest.

Identification of strains. Before purifying the agents for microscopic examination, each strain was propagated in the yolk sac of embryonating eggs and then inoculated into guinea pigs intraperitoneally to obtain immune serum. Blood was collected from each guinea pig at varying intervals after inoculation, and sera obtained from the blood clots were tested by the complement-fixation method of Meyer and Eddie,[10] using the 6BC strain of *Miyagawanella psittaci* as antigen. Confirmation that each yolk preparation contained organisms belonging to the genus *Miyagawanella* was derived from morphologic and tinctorial studies of each strain, serologic evidence based on the development of antibodies in the guinea pigs to the psittacosis-LGV group antigen, and observations of the ability of each strain to produce lesions characteristic of this group of organisms in either mice, guinea pigs, or embryonating eggs, as previously described.

Purification of the strains for electron microscopy. From yolk-sac-propagated suspensions, each strain was adapted by

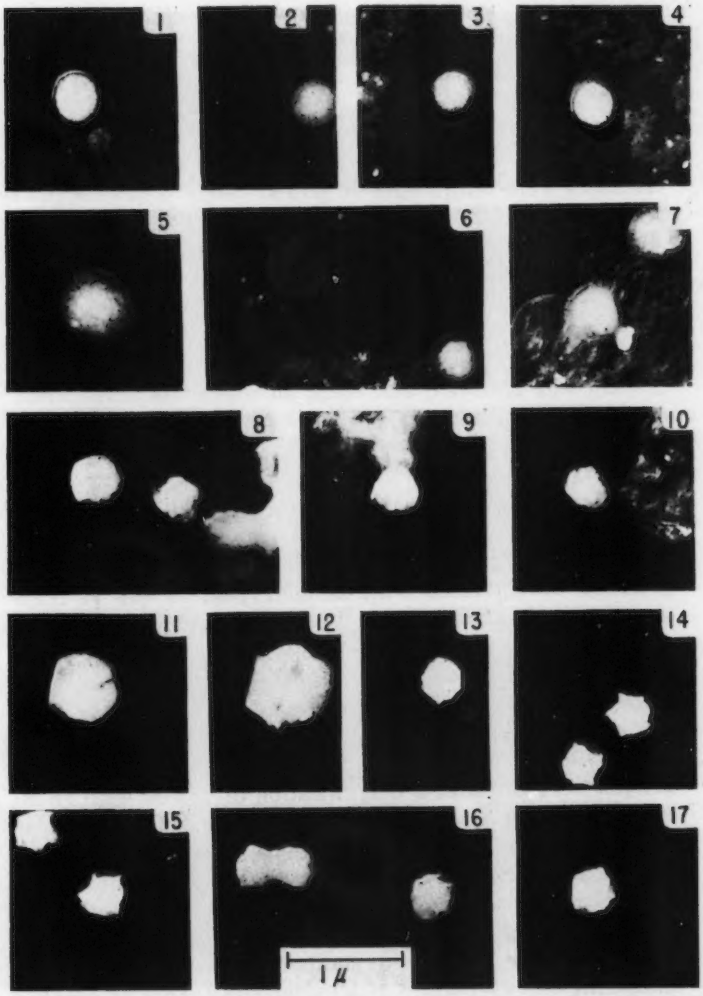


Fig. 1-17. Electron micrographs of Miyagawanella strains (conditions of preparation and photography identical for Figs. 1-43 and 47-52). The micron marker applies to all micrographs on the plate. *1-4)* parakeet strain X1, Hooper Foundation (1955); *5-7)* parakeet strain Pensacola, Hooper Foundation (1955); *8-10)* parakeet strain Robinson, Hooper Foundation (1954); *11-14)* dove strain Dove 33, Hooper Foundation (1951); *15-17)* chicken strain Friend, Karrer *et al.*

serial passage to grow in the chorioallantoic sac of embryonating eggs. The chorioallantoic fluids of embryos dying 4–6 days after inoculation were collected and pooled. As a purity check, samples of infected fluids were stained by Macchiavello's method.[9] Formalin was then added to the pooled fluids to a final concentration of 1%. Within 24 hr of this treatment, the fluids were centrifuged 10 min at 200 × *g* to remove cellular debris, and then for 90 min at 3000 × *g*. The sediment was resuspended in phosphate buffer at pH 8 and subjected to ultrasonic dispersion for 12 min at 400 KC. The suspension of dispersed particles was then treated with trypsin (10 mg/ml, Difco 1:250) for 30 min at 37 C. Cycles of centrifugation and ultrasonic dispersion were repeated until the trypsin was removed, and clean homogeneous suspensions of organisms, as determined by light microscopy after staining, were obtained. Final sediments were suspended in aqueous 1:10,000 merthiolate.

Preparation of strain 13 differed from the above procedure in that the organisms were purified from yolk-sac suspensions rather than from chorioallantoic fluids, these suspensions were treated with chymotrypsin as well as trypsin, and none of the suspensions were subjected to ultrasonic dispersion.

Electron microscopy. A drop of purified suspension was placed on a formvar-coated grid and allowed to air-dry after removing the excess liquid with absorbent tissue. The grid was shadowed with uranium at a 2:1 angle. All micrographs were taken at a magnification of 15,500 with an RCA EMU-3 electron microscope. To select areas for photography more than 50 fields at lower magnifications were examined on each grid. At least 10 areas on each grid were photographed at high magnification to ensure adequate representation of all forms of each strain. Final selection of prints for inclusion in this report was based on photographic clarity without diminishing representation of all sizes and shapes of organisms in each preparation.

RESULTS

Figs. 1–43 show organisms of 13 strains of Miyagawa-

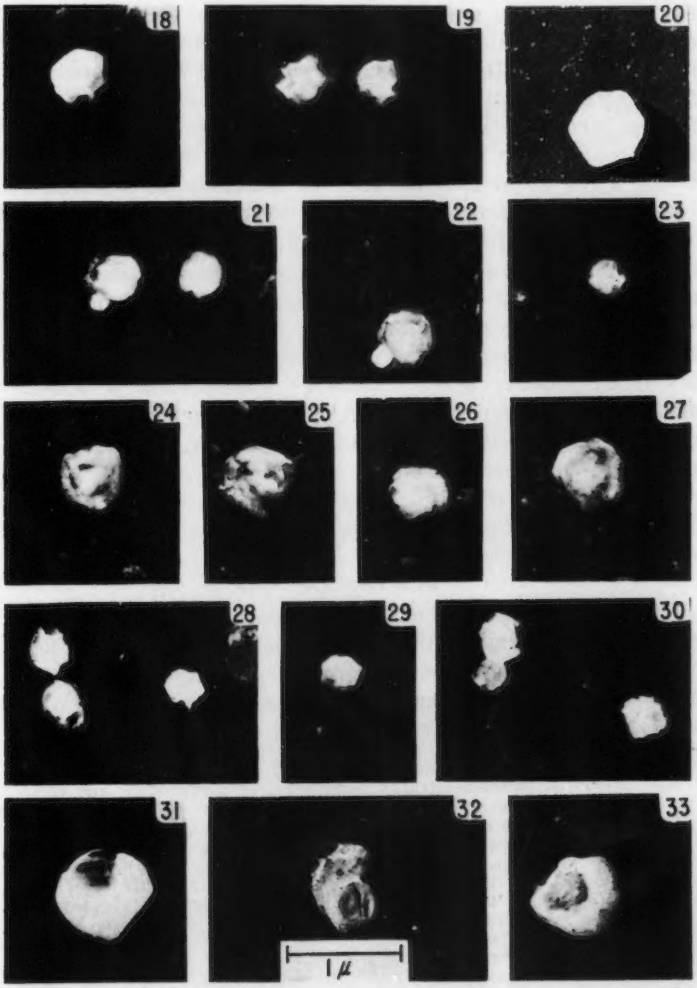


Fig. 18-33. Electron micrographs of Miyagawanella strains (conditions of preparation and photography identical for Figs. 1-43 and 47-52). The micron marker applies to all micrographs on the plate. *18-20)* turkey strain C-1, Bankowski and Page;[2] *21-23)* turkey strain NJ-1, Page;[12] *24-27)* turkey strain NJ-4, Page[13] *28-30)* egret strain, Rubin *et al.*[15] *31-33)* human strain Borg, Olson and Larson.[11]

nella recovered from avian and mammalian (including human) hosts. The photomicrographs revealed the following: 1) No distinct structural features could be correlated with the source of any strain or strains, although elementary bodies of two of three parakeet strains were spherical (Figs. 1–7) whereas many organisms in the remaining strains were angular, suggesting a polygonal architecture. 2) Diameter of organisms in certain strains ranged from 280 to 980 mμ. 3) Envelopes or outer membranes were present on organisms of a majority of the strains. In some cases envelopes separated from the dense central body of the organisms appeared as spherical shells (Fig. 50) or as collapsed membranes (Figs. 47–49, 51, 52). 4) Unshadowed preparations of one agent (Figs. 39, 40) showed an angular outer membrane surrounding a dense, angular central body with small spheres between these two structures. The spheres were 30–70 mμ in diameter and were coincident in size with numerous particles present in the background material of 10 of the preparations (Figs. 3, 16, 21, 24, 36, 41, etc.). These particles are also seen in Fig. 45, from York and Baker.[18]

DISCUSSION

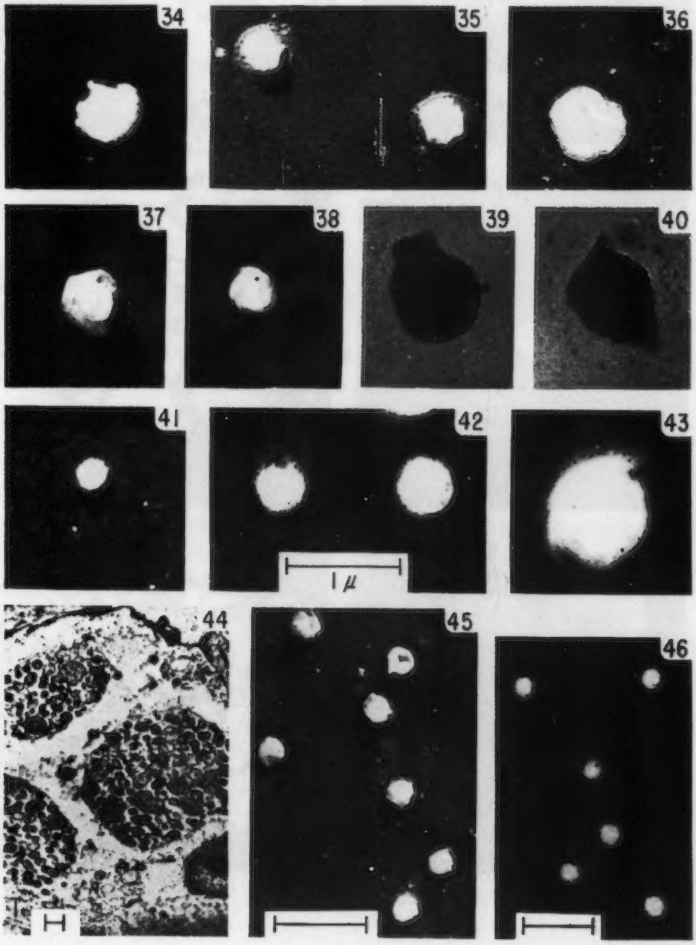
The presence of outer membranes surrounding elementary bodies of Miyagawanella organisms is suggested in published photomicrographs of individual strains,[14,17,18] and evidence presented here establishes their presence. The outer membrane

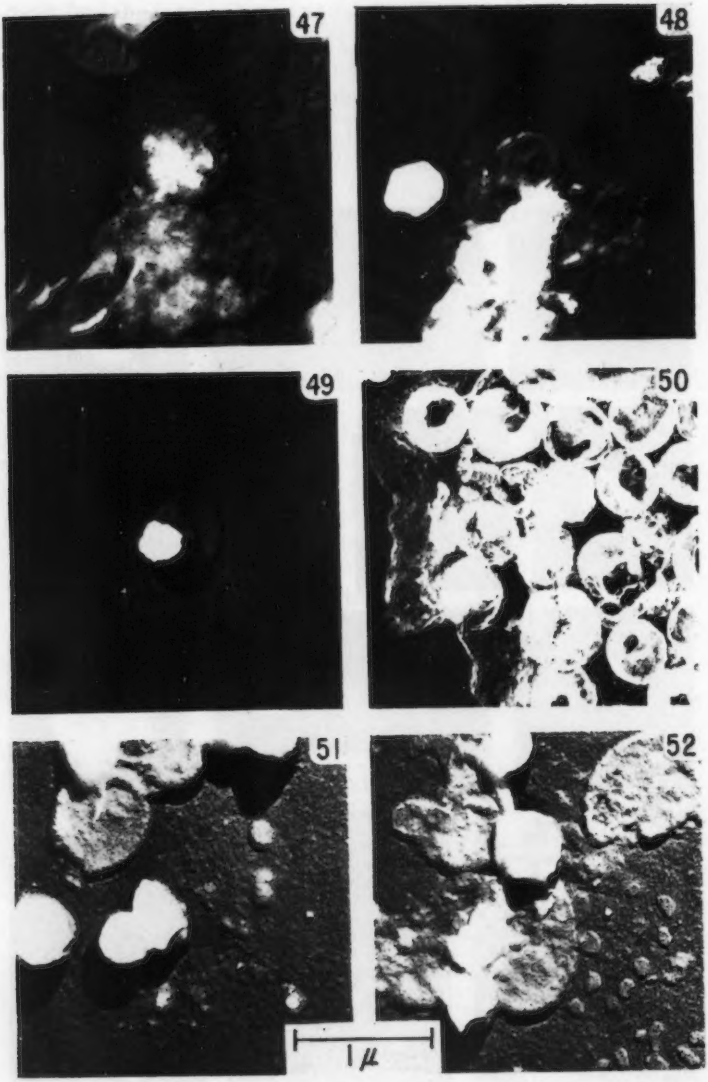
→

Fig. 34–46. Electron micrographs of Miyagawanella strains (conditions of preparation and photography identical for Figs. 1–43 and 47–52). The micron marker applies to all micrographs on the plate except where indicated otherwise (Figs. 44, 45, 46). *34–36)* human (?) strain MP, Francis and Magill;[6] *37-40)* cat strain FP, Baker;[1] *41-43)* EBA strain, Storz *et al.*;[16] *44)* thin section of lung of mouse inoculated 4 days previously with the Maeda strain, showing three discrete inclusion bodies composed mainly of large forms seen in the cytoplasm of an epithelial cell (reprinted by permission of Tajima *et al.*[17] and The Journal of Bacteriology, Williams & Wilkins Co., Baltimore);*45)* *Miyagawanella bovis* (reprinted by permission of York and Baker[18] and the Journal of Experimental Medicine, Rockefeller Press, New York); *46)* "*psittacosis* agent" (reprinted by permission of Roots and Rott[14] and Zentralblatt fur Veterinarmedizin, Paul Parey, Berlin).

with a central body is clearly shown in the unshadowed preparation (Fig 39) as well as in Figs. 47–52, in which envelopes are seen separated from the central body.

Confirmation of the existence of outer membranes on the primary infectious particles of these organisms is of nomenclatural significance. The choice of the prefix *chlamydia* (Gr.,




47
48
49
50
51
52
1 μ

a cloak) in the family name *Chlamydiaceae* Rake, as proposed in Bergey's Manual of Determinative Bacteriology,[3] appears valid.

Aside from nomenclatural considerations, the preparation of homogeneous suspensions of outer membranes and denser central bodies, separated by techniques more refined than reported here, might be useful for detailed studies of the specific and group antigens in these organisms as well as their biochemistry.

The hexagonal appearance (Fig. 20) of many of the individual organisms was not attributed to artifact, since not all the strains exhibited angularity. Elementary bodies of two of the three parakeet strains were decidedly spherical, though the suspensions of these strains were treated like the suspensions of other strains. Hexagonal forms are readily seen in the ultrathin cross sections of psittacosis-infected mouse lung (Fig. 44) photographed by Tajima *et al.*[17] The large forms in Figs. 12, 20, and 43 compare favorably in size and shape with large forms within the intracellular inclusions in Fig. 44. The extraordinary range in size of organisms in the present report may be due to the presence of organisms in various stages of development, as described by Tajima and his co-workers.

Although a majority of the micrographs in this study revealed small background particles ranging in diameter from 30 to 70 mμ, further study is needed to determine whether these particles are related to the particles observed within and about the unshadowed organisms in Figs. 39 and 40, or whether they represent impurities.

←

Fig. 47–52. Electron micrographs of Miyagawanella strains (conditions of preparation and photography identical for Figs. 1–43 and 47–52). The micron marker applies to all micrographs on the plate. *47-48)* parakeet strain X1, Hooper Foundation (1955); *49)* parakeet strain Pensacola, Hooper Foundation (1955); *49)* parakeet strain Pensacola, Hooper Foundation (1955); *50)* turkey strain C-1, Bankowski and Page;[2] *51-52)* dove strain Dove 33, Hooper Foundation (1951).

ADDENDUM

Since this report was submitted, a detailed investigation of the usefulness of cell wall preparations in the study of the serology and biochemistry of one of the agents we photographed was published by H. M. Jenkin ("Preparation and Properties of Cell Walls of the Agent of Meningopneumonitis", J Bact. *80*:639-647, 1960).

ACKNOWLEDGMENTS

Gratitude is expressed to Drs. Bernice U. Eddie and T. T. Crocker, University of California Medical Center, San Francisco, for giving the authors samples of various Miyagawanella strains.

SUMMARY

Structural features of 13 strains of Miyagawanella were compared by electron microscopy. The survey revealed that envelopes or outer membranes were present on elementary bodies of a majority of the strains, and that separated membranes appeared as empty spheres or as collapsed shells. Elementary bodies of two of the strains were spherical, whereas many of the organisms in the other strains exhibited an angularity suggesting a polygonal shape. Diameters of individual organisms ranged from 280 to 980 mμ. Unshadowed organisms contained small, dense spheres, 30–70 mμ in diameter, which were coincident in size with background particles in 10 of the preparations.

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ESCHERICHIA COLI AS A COMPLICATING FACTOR OF NEWCASTLE DISEASE VACCINATION*

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Received 8 July 1960

THE B_1 strain Newcastle disease virus (NDV) was reported by Hitchner and Johnson[3] to be immunogenic, of low virulence, and suitable for use on day-old chicks. They showed that immunity was produced against intramuscular challenge with a neurogenic strain of NDV within 6 days of intranasal vaccination. The strain has since been widely used by the intranasal, intraocular, drinking-water, and aerosol routes as a vaccine for control of Newcastle disease.

Doll *et al.*[1] showed that, though B_1-vaccinated chickens were resistant to intramuscular challenge with a neurogenic strain of NDV, they were susceptible to respiratory tract infection.

During recent years a disease characterized by pericarditis and perihepatitis, called "air sac disease" by some, has caused considerable loss to the poultry industry. Gross[2] showed that the lesions could be reproduced by aerosol exposure of respiratory-disease-infected chickens to certain pathogenic strains of *Escherichia coli*.

This study was made to determine whether the B_1 strain of NDV would promote the production of pericarditis by *E. coli* in vaccinated chicks.

MATERIALS AND METHODS

The chicks used were White Leghorns from the Veterinary Science Department flock. Their parents were free of PPLO

* Supported in part by funds provided by the Animal Disease and Parasite Research Division, A.R.S., U.S.D.A., in the cooperative project on Chronic Respiratory Disease.

and have produced chicks serologically negative and free of PPLO lesions. The breeders were not vaccinated with NDV or infectious bronchitis virus. The chicks were vaccinated at 1, 4 or 7 days of age with the original[3] B_1 strain NDV, propagated in the allantoic sacs of embryonating eggs. One experiment was conducted in 1958, and the second in 1960. The vaccine had a 50% embryo lethal dose (ELD_{50}) of $10^{8.6}$ per ml, in the first experiment, and $5\times10^{8.7}$ per ml in the second experiment. As an aerosol produced by a #40 DeVilbiss nebulizer it was adminstered in chorioallantoic fluid with 5% glycerol to chicks under a 4-cubic-foot plastic box. Aerosol exposure with an undiluted broth culture of a pathogenic strain of *E. coli* (1-1 serotype) was made 2, 4, 8, 16, or 22 days after vaccination. At the same time, groups of unvaccinated birds were also exposed. The exposed chicks were housed in modifed Horsfall-Bauer units with thermostatically controlled heaters. Five to eight days after coliform exposure the chicks were examined for pericarditis. The numbers of chicks vaccinated and/or exposed to *E. coli* are shown in Table 1.

RESULTS

The aerosol-administered vaccines of high titer with 5% glycerol caused respiratory reactions with mortalities of 10% in the first experiment and 4% in the second. Respiratory signs

Table 1. The incidence of pericarditis in Newcastle-disease-vaccinated chickens when exposed to aerosols of *E. coli* at various times after vaccination.

Period between vaccination and exposure (days)	Age when vaccinated for Newcastle Disease				
	1st experiment		2nd experiment		
	1 day	4 days	1 day	4 days	7 days
2	1/8[1]	2/10	0/10	6/10	0/10
4	2/8	3/10	9/12	4/12	1/10
8	5/5	5/9	5/13	6/14	5/13
16	2/6	3/10	4/11	5/18	2/16
22		1/10	2/13	0/12	2/14
Unvaccinated controls		0/30	0/35		

[1] Numerator, number with pericarditis; denominator, number exposed.

with much gaping were seen between the 2nd and 18th days after vaccination. After *E. coli* aerosol exposure the chicks became slightly to moderately depressed and a few died, particularly in the groups exposed 2 days after vaccination. The incidence of pericarditis following *E. coli* exposure appears in Table 1. These results indicate that *E. coli* was able to invade the vaccinated birds but not the unvaccinated. Susceptibility was greatest in chickens vaccinated at 1 or 4 days of age. Susceptibility was greatest 8 days post-vaccination, and continued to at least 22 days post-vaccination.

The results indicate that the mild B_1 strain of NDV does make chicks susceptible to areosols of pathogenic strains of *E. coli*, and that this susceptibility lasts until at least 22 days post-vaccination. After the 6th day post-vaccination, the chicks should have been immune[3] to systemic Newcastle disease, which seems to indicate that increased susceptibility to coliforms is the reaction of the respiratory tract alone. The reduced susceptibility to *E. coli* of chicks vaccinated at 7 days suggests that susceptibility induced by B_1 NDV infection might occur chiefly in young birds. "Air sac disease" outbreaks might follow similar vaccine reactions in the field. These results suggest that killed NDV vaccines might be much safer than live vaccines. It should be determined whether other Newcastle disease vaccines can produce similar reactions.

SUMMARY

Pericarditis could be produced by exposing chicks vaccinated 2–22 days previously with B_1 Newcastle disease virus to aerosols of pathogenic strains of *Escherichia coli*. This could not be done with unvaccinated chicks.

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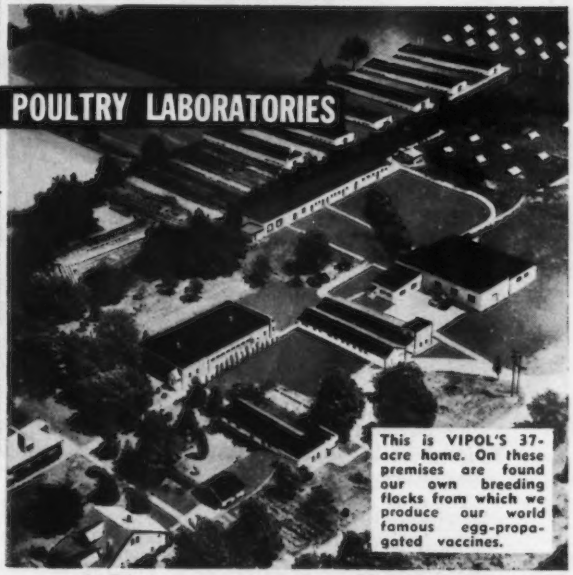

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and for chronic respiratory disease (C. R. D.) of poultry . . .

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2. Shumard, R. F.: Paper presented at the First National Symposium on Nitrofurans in Agriculture, Michigan State University, East Lansing, Michigan, Sept. 28-29, 1956.

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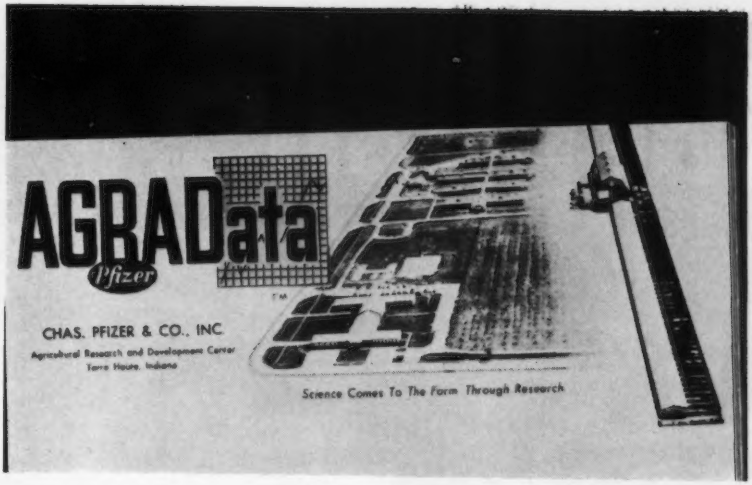

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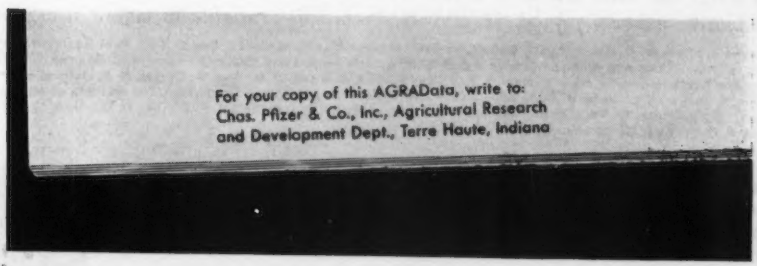
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SERPASIL has been tested in repeated field trials throughout this country and Canada over a 2-year period. SERPASIL Premix is available to veterinarians now.

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- **CARB-O-SEP**—the superiority of CARB-O-SEP in effectively preventing blackhead in turkeys has been well proven in experimental use at the college experiment station level—in tests by feed manufacturers—and in regular farm use. Maximum weight gains and top feed efficiency are other important advantages shown by CARB-O-SEP fed birds.
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*Confirming reports by independent workers on the synergism that exists in WHITSYN-type drug combinations have been published in several journals. Data will be supplied upon request.

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WEIGHT, FEED EFFICIENCY, FINISH, AND MORTALITY DATA

Lot Number	1B	2B	3B	4B	5B	6B	7B	8B
Drug Treatment	None	1 lb. Histostat 50	1 lb. 11 oz. Hepzide 30	2 lb. 5 oz. Hepzide 30	2 lb. NF 64	3 lb. NF 64	3 lb. NF 179	2 lb. NF 180
Average Weight Toms, Lbs.	21.96	22.94	21.81	21.95	22.47	21.49	23.44	23.05
Average Weight Hens, Lbs.	15.13	15.58	14.99	15.01	15.72	15.28	15.54	14.74
Feed Required Per Lb. Gain	5.08	4.17	4.67	4.32	4.63	3.91	3.98	4.48
Feathering—Toms.	93.75	98.75	94.44	100.00	100.00	89.29	98.96	97.50
Hens	100.00	100.00	100.00	100.00	98.75	98.86	100.00	99.00
Fleshing —Toms.	84.38	96.25	90.28	84.38	92.30	80.95	92.71	88.75
Hens	100.00	100.00	100.00	100.00	98.75	98.86	100.00	98.00
Mortality Blackhead, %	24%	2%	12%	10%	22%	14%	4%	12%

Please note the consistent superiority of lot 2B (Histostat-50). This lot had the *lowest death loss*, and was *highest of all the commercially available drugs tested on weight gains, feed efficiency*, and *fleshing*.

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